National Toxicology Program
Toxicity Report Series
Number 81

NTP Technical Report on the Toxicity Studies of

α-Pinene

(CAS No. 80-56-8)

Administered by Inhalation to F344/N Rats and B6C3F1/N Mice

May 2016

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

FOREWORD

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National Institutes of Health
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PEER REVIEW

The draft report on the toxicity studies of α-pinene was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that this Toxicity Study Report presents the experimental results and conclusions fully and clearly.

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CONTENTS

SUMMARY

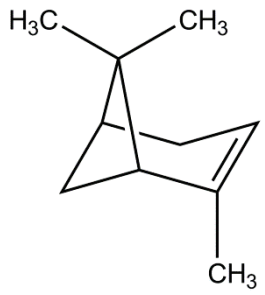
Background
α-Pinene is the main component of turpentine and is also used as a fragrance in cleaning products and perfumes. We studied the effects of α-pinene on male and female rats and mice to identify potential toxic hazards to humans.

Methods
We exposed groups of 10 male and 10 female rats and mice to atmospheres containing α-pinene vapors at concentrations of 25, 50, 100, 200, or 400 parts per million of air (ppm) for 6 hours per day, 5 days per week for 3 months. Other groups were not exposed to the chemical and served as controls. Tissues from more than 40 sites were examined for every animal in the control and top dose groups.

Results
Six of the 10 female rats exposed to 400 ppm α-pinene died before the end of the study; all other animals survived. Liver weights were increased relative to controls for male and female rats and mice exposed to the highest concentration of α-pinene, and increased kidney weights and associated lesions of the kidney were seen in male rats. Increases of transitional epithelium hyperplasia of the urinary bladder were noted in male and female mice exposed to 100 ppm or greater concentrations of α-pinene. Male rats and male mice exposed to the two highest concentrations of α-pinene had reduced numbers of sperm per cauda.

Conclusions
We conclude that α-pinene caused increased liver weights in male and female rats and mice, urinary tract lesions (kidney of rats and urinary bladder of mice), and reduction in cauda epididymal sperm in male rats and mice.

ABSTRACT



α-PINENE

CAS No. 80-56-8

Chemical Formula: $C_{10}H_{16}$ Molecular Weight: 136.24

Synonyms: Acitene A; cyclic dexadiene; 2-pinene; 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene

α-Pinene is the main component in turpentine and is used as a fragrance and flavoring ingredient. Due to widespread exposure potential and a lack of available toxicity data, male and female F344/N rats and B6C3F1/N mice were exposed to α-pinene (96% pure) by inhalation for 2 weeks or 3 months. Genetic toxicology studies were conducted in *Salmonella typhimurium*, *Escherichia coli*, and mouse peripheral blood erythrocytes.

In the 2-week studies, groups of five male and five female rats and mice were exposed to α-pinene by whole body inhalation at concentrations of 0, 100, 200, 400, 800, or 1,600 ppm, 6 hours plus T_{90} (12 minutes) per day, 5 days per week for 16 (rats) or 17 (mice) days. There was significantly decreased survival in the 800 and 1,600 ppm male and female rats and mice, clinical signs of toxicity in rats exposed to 400 ppm or greater and mice exposed to 800 or 1,600 ppm, and increased liver weights (up to 21%) in both species. Histopathologic lesions noted in the 2-week studies were confined to minimal olfactory epithelial degeneration of nasal tissue in male and female mice exposed to 800 and 1,600 ppm (data not presented).

In the 3-month studies, groups of 10 male and 10 female rats and mice were exposed to α-pinene by whole body inhalation at concentrations of 0, 25, 50, 100, 200, or 400 ppm, 6 hours plus T_{90} (10 minutes) per day, 5 days per week for 14 weeks. All exposed male rats and male and female mice survived to the end of the studies, while six 400 ppm female rats died before the end of the study The major targets for α-pinene toxicity were the liver, urinary system, and male reproductive system. The absolute liver weights were significantly greater than those of the chamber controls in 400 ppm male rats (13%), male mice (21%), and female mice (18%), and female rats exposed to 50, 100, or 200 ppm (14%, 14%, and 17%, respectively); however, accompanying treatment-related histopathologic lesions did not occur

in the liver of male or female rats or mice. Absolute kidney weights were increased in male rats exposed to 100 ppm or greater (up to 25%) and 50 and 200 ppm female rats (10%); in males, these increases were accompanied by histopathologic lesions including granular casts and hyaline droplet accumulation at all exposure concentrations, as well as exposure concentration-dependent increases in the severity of nephropathy, which is a common spontaneous lesion observed in male rats. Exposure concentration-dependent increased incidences of transitional epithelium hyperplasia of the urinary bladder occurred in male and female mice exposed to 100 ppm or greater (males: 100 ppm, 70%; 200 ppm, 100%; 400 ppm, 100%; females: 60%, 100%, 100%). There were also significantly lower numbers of sperm per cauda compared to the chamber controls in 200 and 400 ppm male rats (19%) and 100, 200, and 400 ppm male mice (24%, 33%, and 40%, respectively).

α-Pinene was not mutagenic in *S. typhimurium* strains TA98 or TA100 or in *E. coli*, with or without exogenous metabolic activation. No increase in micronucleated erythrocytes was seen in male or female mice in the 3-month study.

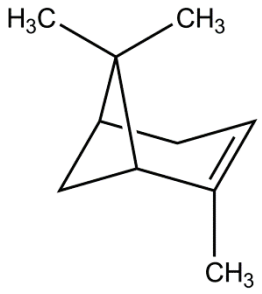
The current permissible exposure limit and recommended airborne exposure limit for α-pinene is 100 ppm (as turpentine) and the threshold limit value is 20 ppm averaged over an 8-hour workshift.

Under the conditions of the 3-month inhalation studies, there were treatment-related lesions in male and female rats and mice. The major targets from α-pinene exposure in rats and mice included the liver, urinary system (kidney of rats and urinary bladder of mice), and cauda epididymal sperm. The most sensitive measures of α-pinene exposure in each species and sex were increased incidences of kidney lesions in male rats [lowest-observed-effect level (LOEL)=25 ppm], increased relative liver weights in female rats (LOEL=25 ppm) without accompanying histopathologic changes, decreased sperm per cauda and increased incidences of transitional epithelium hyperplasia of the urinary bladder in male mice (LOEL=100 ppm), and increased incidences of transitional epithelium hyperplasia of the urinary bladder in female mice (LOEL=100 ppm).

Summary of Findings Considered to be Toxicologically Relevant in Rats and Mice Exposed to α-Pinene by Inhalation for 3 Months

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
Concentrations in air	0, 25, 50, 100, 200, or 400 ppm	0, 25, 50, 100, 200, or 400 ppm	0, 25, 50, 100, 200, or 400 ppm	0, 25, 50, 100, 200, or 400 ppm
Survival rates	10/10, 10/10, 10/10, 10/10, 10/10, 10/10	10/10, 10/10, 10/10, 10/10, 10/10, 4/10	10/10, 10/10, 10/10, 10/10, 10/10, 10/10	10/10, 10/10, 10/10, 10/10, 10/10, 10/10
Body weights	Exposed groups similar to the chamber control group	400 ppm group 18% less than the chamber control group	Exposed groups similar to the chamber control group	Exposed groups similar to the chamber control group
Clinical findings	None	None	None	None
Organ weights	↑ Absolute and relative kidney weights; ↑ Absolute and relative liver weights	↑ Absolute and relative heart weights; ↑ Absolute and relative kidney weights; ↑ Absolute and relative liver weights	↓ Absolute kidney weights; ↑ Absolute and relative liver weights	↑ Absolute and relative liver weights
Clinical pathology	None	None	None	None
Reproductive toxicity	↓ Sperm per cauda	None	↓ Sperm per cauda	None
Nonneoplastic effects	Kidney: granular casts (0/10, 9/10, 10/10, 10/10, 10/10, 10/10); hyaline droplet accumulation (1/10, 10/10, 10/10, 10/10, 10/10, 10/10)	None	Urinary bladder: transitional epithelium hyperplasia (0/10, 0/10, 0/10, 7/10, 10/10, 10/10)	Urinary bladder: transitional epithelium hyperplasia (0/10, 0/10, 0/10, 6/10, 10/10, 10/10)
Genetic toxicology				
Bacterial gene mutations:	Negative in *E. coli* with or without S9; negative in *S. typhimurium* strains TA98 and TA100 with or without S9			
Micronucleated erythrocytes				
Mouse peripheral blood *in vivo*:	Negative in males and females			

INTRODUCTION



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CHEMICAL AND PHYSICAL PROPERTIES

α-Pinene is a bicyclic monoterpene emitted from plant matter and exists as a colorless, oily liquid with a strong odor (HSDB, 2010). α-Pinene is present in conifer trees and turpentine as a mixture of (+) and (–) enantiomers, which can differ in ratio according to species, source tissue (e.g., needles, xylem), and age (Persson *et al*., 1996; Sjödin *et al*., 1996; Wibe *et al*., 1998; Phillips *et al*., 1999). There is enantiospecificity to the odor of *α*-pinene, with (+)-α-pinene producing a slightly minty odor and (–)-α-pinene producing a pine scent (de Carvalho and da Fonseca, 2006). α-Pinene has a density of 0.859 at 20° C relative to water at 4° C, a boiling point of 155° to 156° C at 760 mm mercury, and a refractive index for sodium light of 1.466 at 20° C (*Merck*, 2006). α-Pinene is relatively hydrophobic with a measured solubility in water of 18 µg/mL at 20° C and a calculated logP value of 4.37 (Cal, 2006).

Monoterpenes such as α-pinene belong to the terpenoid class of chemicals, which are based on isoprene units and represent the largest group of naturally occurring compounds with over 22,000 terpenoids identified (McGarvey and Croteau, 1995). Structurally related monoterpenes include d-limonene and Δ^3-carene.

α-Pinene is a volatile organic compound that can react with nitric oxide to form ozone in the troposphere (Atkinson and Arey, 2003). Major pathways of removal and transformation of α-pinene from the atmosphere include reactions with hydroxyl radical, nitrate radical, or ozone. Products formed from these reactions are pinonaldehyde, acetone, formaldehyde, formic acid, and hydroxyl radical, among others (Atkinson and Arey, 2003).

PRODUCTION, USE, AND HUMAN EXPOSURE

α-Pinene, produced by pine trees and various other plants, is the main component of turpentine. Although α-pinene is ubiquitous due to its volatilization from pine trees, there are two potential pathways that lead to more significant α-pinene exposure in humans: 1) processing, use, or storage of softwoods or their by-products (e.g., turpentine), and 2) use of personal care products, cleaning products, or air fresheners containing α-pinene as a fragrance component. Measured concentrations of α-pinene occupy a wide range from tens of μg/m^3 to hundreds of mg/m^3. The current permissible exposure limit and recommended airborne exposure limit for α-pinene is 100 ppm (as turpentine) (NIOSH, 2010) and the threshold limit value is 20 ppm averaged over an 8-hour workshift (ACGIH, 2015).

Several studies have measured the levels of α-pinene or combined terpenes in the lumber industry. Demers *et al.* (2000) measured α-pinene concentrations in Canadian softwood lumber mills using personal passive sampling devices and reported a geometric mean of 0.1 mg/m^3 (geometric SD = 3.8 mg/m^3; approximately 0.018 ± 0.68 ppm). New Zealand plywood workers were exposed to α-pinene concentrations of 0.5 to 2.4 mg/m^3 (approximately 0.090 to 0.43 ppm) (Fransman *et al.*, 2003). Significantly higher α-pinene levels were detected in Finnish sawmills, in the range of 57 to 152 mg/m^3 (approximately 10 to 27 ppm) (Rosenberg *et al.*, 2002). Personal exposure to the monoterpenes α-pinene, β-pinene, and Δ^3-carene ranged from 10 to 214 mg/m^3 (approximately 1.8 to 38 ppm) in Swedish joinery shops (Eriksson *et al.*, 1997) and 0.64 to 28 mg/m^3 (approximately 0.11 to 5.0 ppm) in Swedish wood pellet manufacturing facilities (Edman *et al.*, 2003). The highest levels of terpenes reported were in Swedish lumber mills and ranged from 100 to 500 mg/m^3 (approximately 18 to 90 ppm), with an average of 254 mg/m^3 (approximately 46 ppm) (Hedenstierna *et al*., 1983). Concentrations of α-pinene ranging from 0.078 to 0.333 mg/m^3 (approximately 0.014 to 0.060 ppm) were measured in the blower exhaust from a composting facility (Van Durme *et al*., 1992).

α-Pinene is used as a fragrance component in perfumes, air fresheners, personal care products, and household cleaners (Nazaroff and Weschler, 2004). Rastogi *et al.* (2001) detected α-pinene in 39% of 59 occupational and domestic products tested with a mean concentration of 41.3 ± 51.5 ppm. A maximum concentration of α-pinene measured in a small chamber following application of floor wax containing the chemical as a major component was 0.0683 mg/m^3 (approximately 0.012 ppm) (Colombo *et al*., 1991). α-Pinene is also a common contaminant detected in indoor air samples. In one review of indoor air quality, mean concentrations of α-pinene reported were 4 to 10 mg/m^3 (approximately 0.72 to 1.8 ppm), with maximum concentrations of 120 to 208 mg/m^3 (approximately 22 to 37 ppm) (Namieśnik *et al*., 1992).

ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION, AND TOXICOKINETICS

In general, data from human exposure to α-pinene have demonstrated that it is rapidly absorbed after inhalation exposure, accumulates in the fat compartment, is metabolized primarily by hydroxylation and glucuronidation, and is excreted by the kidneys (Falk *et al*., 1990; Filipsson, 1996).

The uptake, distribution, and elimination of α-pinene was investigated in healthy male volunteers following a 2-hour inhalation exposure to 10, 225, and 450 mg/m^3 (approximately 1.8, 40, and 81 ppm) (+)-α-pinene or 450 mg/m^3 (–)-α-pinene (Falk *et al*., 1990). Significant differences were not found between the enantiomers in uptake, distribution, or excretion. α-Pinene exhibited a high degree of uptake from the lungs, averaging 59% for the two higher concentrations, with approximately 8% of the parent compound eliminated in exhaled air. Less than 0.001% of the total uptake was eliminated unchanged in the urine. Saturation of metabolism was not observed, as evidenced by a linear increase in arterial blood concentration with increasing exposure concentration. The clearance value for α-pinene indicated that it was readily metabolized. A tri-phasic elimination curve was observed with half-lives for each phase of elimination equal to 4.8 and 5.6, 38 and 40, and 695 and 555 minutes, respectively, for (+)- and (–)-α-pinene. The long half-life associated with the third phase of elimination indicates a high affinity of α-pinene for poorly perfused tissue (i.e., adipose tissue).

Animal metabolism studies in the rabbit and brushtail possum support the metabolic pathway identified in the human studies and have identified many of the same α-pinene metabolites, with the verbenols representing the major metabolites and myrtenol and myrtenic acid in lesser amounts (Southwell *et al*., 1980; Ishida *et al*., 1981). Metabolism of inhaled α-pinene occurs mainly via hydroxylation and glucuronidation followed by renal elimination (Levin *et al.*, 1992). The major metabolites *cis*- and *trans*-verbenol, representing approximately 1% to 4% of the total uptake, were identified in the urine of experimentally exposed individuals. These urinary metabolites, in addition to trace amounts of the metabolite myrtenol, were also identified in mill workers exposed occupationally to α-pinene, β-pinene, and Δ^3-carene (Eriksson and Levin, 1990).

In contrast to the profile of absorption, distribution, metabolism, and elimination following inhalation exposure, a case study describing the fate of monoterpenes following an intentional ingestion of pine oil (57% α-pinene, 8% β-pinene, 26% Δ^3-carene, 6% limonene, and 3% other) found poor uptake of the monoterpenes from the gastrointestinal tract, slow metabolism, and renal excretion of metabolites (Köppel *et al*., 1981). The major metabolites identified for α-pinene, borneol and bornylacetate, also differed from those identified following inhalation.

TOXICITY

In humans, reports of toxicity resulting from α-pinene alone or terpene mixtures containing α-pinene indicate potential respiratory and skin irritation. Johard *et al*. (1993) assessed the effects of short-term inhalation exposure to a terpene mixture (α-pinene, β-pinene, and Δ^3-carene) on bronchioalveolar lavage fluid from eight healthy volunteers and found that macrophage and mast cell counts increased following exposure to 450 mg/m^3. Irritation of the eyes, nose, and throat was observed in volunteers exposed to 450 mg/m^3 α-pinene (Falk *et al*., 1990).

Animal studies designed to address the general toxicity, reproductive toxicity, or developmental toxicity of α-pinene were not found in the literature. α-Pinene elicited sensory irritation (stimulation of specific nerve endings in the nasolaryngeal region leading to characteristic “braking” during exhalation and a corresponding decrease in respiratory

frequency) in a mouse bioassay with a concentration that reduces respiratory rate by 50% (RD_{50}) of 1,053 to 1,107 ppm for the more active D-α-pinene enantiomer (Kasanen *et al*., 1998). α-Pinene was positive in an acute dermal irritation assay and negative in a guinea pig maximization test, indicating that it is a skin irritant but not a sensitizer (Wei *et al.*, 2006).

CARCINOGENICITY

Very little carcinogenicity data are available for α-pinene. Two epidemiological studies have examined turpentine or terpene exposure in occupational settings and cancer outcomes. In a case-control study of Finnish woodworkers, a weak association [odds ratio (OR) = 1.33; 95% confidence interval (CI): 0.78, 2.27 for any exposure to terpenes lasting over one month] was found between respiratory cancer and exposure to terpenes (primarily α-pinene and Δ^3-carene) and other heating products of pine and spruce (Kauppinen *et al*., 1993). Another case-control study found an association between paternal exposure to turpentine and neuroblastoma in offspring (OR = 1.9; CI: 1.0, 3.6 to 10.4; CI: 2.4, 44.8 depending on methods for exposure categorization) (De Roos *et al*., 2001). Chronic toxicity studies with *α*-pinene were not found in the literature.

GENETIC TOXICITY

Genotoxicity studies of α-pinene indicated that it was not positive in bacterial mutagenicity assays but demonstrated clastogenic and aneugenic effects in one *in vitro* mammalian cell study.

α-Pinene was not mutagenic in several bacterial mutagenicity assays. α-Pinene, tested at a single concentration of 3 µM, was negative in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 with or without Arochlor 1254-induced rat liver S9 metabolic activation enzymes, and α-pinene tested over concentrations that ranged up to 3 µM was negative in *S. typhimurium* strains TA98 and TA100 with or without 3-methylcholanthrene-induced rat liver S9 mix (Florin *et al*., 1980). Additionally, α-pinene was negative in *S. typhimurium* strains TA98 and TA100 when tested at concentrations ranging from 10 to 500 µg/plate with or without S9 mix (Connor *et al*., 1985), and enantiomers of α-pinene were negative in *S. typhimurium* strains TA97a, TA98, TA100, and TA1535 at concentrations ranging from 100 to 5,000 µg/plate with or without S9 mix (Gomes-Carneiro *et al*., 2005).

Two *in vitro* genotoxicity studies of α-pinene were performed using mammalian cells. α-Pinene did not induce DNA damage as assessed by the comet assay in human lung A549 cells in a system that allowed exposure to α-pinene by air (concentrations ranged from 1 to 1,800 mg/m^3) (Gminski *et al*., 2010). However, α-pinene was clastogenic and aneugenic in V79-C13 Chinese hamster cells exposed in cell culture medium (Catanzaro *et al*., 2012). Clastogenic activity was evidenced by induction of DNA damage assessed by the comet assay, significant increases in micronucleated cells, and induction of chromosomal breakage assessed by metaphase analysis. With regard to the mechanism of DNA damage, α-pinene generated significant increases in reactive oxygen species as measured by a fluorescence assay. Furthermore, a significant number of the micronuclei observed in the V79-C13 cells stained

positive for the presence of kinetochores, the number of chromosomes in metaphase spreads deviated from the modal number (decreased with increasing concentrations of α-pinene), and a significant increase in metaphase spreads showing endoreduplication was noted, suggesting that α-pinene has aneugenic activity. Immunofluorescent detection of tubulin and counterstaining for chromatin showed that the mitotic spindle was disrupted in cells exposed to α-pinene. Although concentrations of α-pinene that ranged from 40 to 50 μM induced very high levels of apoptosis, the clastogenic and aneugenic effects of α-pinene were observed at concentrations ranging from 25 to 35 μM that were accompanied by low levels of apoptosis.

STUDY RATIONALE

Originally, turpentine was nominated by the International Union of the United Auto Workers for comprehensive toxicity studies due to widespread human exposure and a lack of data characterizing the chronic effects associated with turpentine. The National Toxicology Program proceeded with testing the main component of turpentine, α-pinene, which has a more diverse and widespread exposure profile. In addition to being the main component in turpentine, α-pinene is also used as a fragrance and flavoring ingredient. Exposure to α-pinene occurs through the use of personal care and cleaning products, as well as occupationally, in lumber processing and building activities. Furthermore, the toxicity data available for α-pinene are inadequate for assessing potential human health effects. This Toxicity Study Report summarizes the results of 2-week and 3-month inhalation toxicity studies with α-pinene in F344/N rats and B6C3F1/N mice.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF α-PINENE

α-Pinene was obtained from Millennium Specialty Chemicals (Jacksonville, FL) in one lot (4KB705) that was used in the 2-week and 3-month studies. Identity and purity analyses were conducted by the study laboratory at Battelle Toxicology Northwest (Richland, WA), Chemir Analytical Services (Maryland Heights, MO), Galbraith Laboratories, Inc. (Knoxville, TN), and Huffman Laboratories, Inc. (Golden, CO) (Appendix F). Reports on analyses performed in support of the α-pinene studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a colorless oily liquid with a strong piney odor, was identified as α-pinene by Chemir Analytical Services using infrared and ^{1}H-nuclear magnetic resonance spectroscopy. All spectra were consistent with the literature reference spectra.

Karl Fischer titration indicated a water content of 27 ppm. Elemental analyses for carbon, hydrogen, nitrogen, and sulfur were in agreement with the theoretical values for α-pinene. Gas chromatography with flame ionization detection (GC/FID) indicated one major peak accounting for approximately 96% of the total integrated peak area and three impurity peaks with areas exceeding 0.1% of the total peak area; two of these peaks matched the retention times for prepared standards of camphene (1.77%) and β-pinene (1.73%). Gas chromatography with mass spectrometry (GC/MS) identified the third impurity as tricyclene (0.51%). Enantiomeric composition analysis using GC/FID with a chiral separation column indicated that the lot was 69% (+)-α-pinene and 31% (–)-α-pinene. The overall purity of the lot was determined to be approximately 96%. Analysis using GC/MS indicated that approximately 15 to 16 ppm butylated hydroxy toluene, a free radical scavenger, was present in the lot to prevent oxidation of α-pinene.

To ensure stability, the bulk chemical was stored at 17° C in the original shipping containers (55-gallon metal drums). Periodic reanalyses of the bulk chemical were performed during the 2-week and 3-month studies by the study laboratory using GC/MS, and no degradation of the bulk chemical was detected.

VAPOR GENERATION AND EXPOSURE SYSTEM

The vapor transport lines and all dilution air were heated, and α-pinene was pumped through a preheater (for the 2-week studies) and into a heated glass column filled with glass beads that increased the surface area for vaporization. Heated nitrogen entered the column from below and assisted in vaporizing the chemical while conveying it into a short distribution manifold. Concentration in the manifold was determined by the chemical pump rate, nitrogen flow

rate, and dilution air flow rate. The pressure in the distribution manifold was kept fixed to ensure constant flow through the manifold and into all chambers as the flow of vapor to each chamber was adjusted.

Metering valves at the manifold controlled flow to each chamber through individual Teflon® delivery lines that carried the vapor from the manifold to three-way exposure valves at the chamber inlets. The exposure valves diverted vapor delivery to exposure chamber exhaust until the generation system was stable and exposures were ready to proceed. To initiate exposure, the chamber exposure valves were rotated to allow the α-pinene vapor to flow to each exposure chamber inlet duct where it was further diluted with filtered, conditioned air to achieve the desired exposure concentration.

The study laboratory designed the inhalation exposure chamber (Lab Products, Inc., Seaford, DE) so that uniform vapor concentrations could be maintained throughout the chamber with the catch pans in place. The total active mixing volume of each chamber was 1.7 m^3. A condensation particle detector was used with and without animals in the exposure chambers. No particle counts above the minimum resolvable level (approximately 200 particles/cm^3) were detected.

VAPOR CONCENTRATION MONITORING

Summaries of the chamber vapor concentrations are given in Tables F2 and F3. Chamber and room concentrations of α-pinene were monitored by an on-line gas chromatograph. Samples were drawn from each exposure chamber approximately every 20 minutes during each 6-hour exposure period. A 16-port stream select valve directed a continuous stream of sampled atmosphere to a six-port sampling valve with a sample loop, housed in a dedicated valve oven. A vacuum regulator maintained a constant vacuum in the sample loop to compensate for variations in sample line pressure. An in-line flow meter between the vacuum regulator and the gas chromatograph allowed digital measurement of sample flow. The on-line gas chromatograph was checked throughout the day for instrument drift against an on-line standard vapor of α-pinene in nitrogen supplied by a standard generator. The on-line gas chromatograph was recalibrated as required to meet acceptance criteria. Calibration was performed by a comparison of chamber concentration data to data from grab samples that were collected with activated coconut charcoal gas sampling tubes, extracted with toluene containing butylbenzene as an internal standard, and analyzed using an off-line gas chromatograph. Known volumes of chamber atmosphere were sampled at a constant flow rate ensured by a calibrated critical orifice. The off-line gas chromatograph was calibrated with gravimetrically prepared standards of α-pinene containing butylbenzene as an internal standard in toluene.

CHAMBER ATMOSPHERE CHARACTERIZATION

Buildup and decay rates for chamber vapor concentrations were determined with and without animals present in the chambers. At a chamber airflow rate of 15 air changes per hour, the theoretical value for the time to achieve 90% of the target concentration after the beginning of vapor generation (T_{90}) and the time for the chamber concentration to

decay to 10% of the target concentration after vapor generation was terminated (T_{10}) was approximately 9.4 minutes. T_{90} values of 12 and 10 minutes were selected for the 2-week and 3-month studies, respectively.

Evaluations of chamber uniformity and persistence and monitoring for α-pinene degradation impurities were conducted periodically throughout the studies by gas chromatography. Chamber uniformity was maintained; no degradation was detected.

ANIMAL SOURCE

Male and female F344/N rats and B6C3F1/N mice were obtained from the NTP colony maintained at Taconic Farms, Inc. (Germantown, NY), for the 2-week and 3-month studies.

2-WEEK STUDIES

On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 11 days and were 6 weeks old on the first day of the studies. Before the studies began, five extra male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female chamber control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix H). Groups of five male and five female rats and mice were exposed to α-pinene via whole body inhalation at concentrations of 0, 100, 200, 400, 800, or 1,600 ppm, 6 hours plus T_{90} (12 minutes) per day, 5 days per week for 16 (rats) or 17 (mice) days. Concentrations were selected based on studies of turpentine toxicity performed by Chapman (1941) that involved exposure of rats to roughly estimated (by the NTP) concentrations of 5,000 to 10,000 mg/m^3 (897 to 1,795 ppm) turpentine via inhalation for 6.5 to 293 total hours over periods ranging from 5 days to 14 months with no chemical-related lesions observed in the kidneys. Feed was available *ad libitum* except during exposure periods; water was available *ad libitum*. Rats and mice were housed individually. Clinical findings were recorded twice daily on exposure days for rats and mice. The animals were weighed initially, on days 6 and 13, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all rats and mice. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Histopathologic examinations were performed on all chamber control, 400, 800 and 1,600 ppm rats and mice. Table 1 lists the tissues and organs examined.

3-MONTH STUDIES

On receipt, the rats and mice were approximately 4 weeks old. Animals were quarantined for 12 (male rats and male and female mice) or 13 (female rats) days and were 5 to 6 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation

for evidence of disease. Serologic analyses were performed on five male and five female sentinel rats and mice at 1 week and at the end of the studies using the protocols of the NTP Sentinel Animal Program (Appendix H).

Groups of 10 male and 10 female rats and mice were exposed to α-pinene via whole body inhalation at concentrations of 0, 25, 50, 100, 200, or 400 ppm, 6 hours plus T_{90} (10 minutes) per day, 5 days per week for 14 weeks. Groups of 10 male and 10 female clinical pathology rats were exposed to the same concentrations for 23 days. Feed was available *ad libitum* except during exposure periods; water was available *ad libitum*. Rats and mice were housed individually throughout the study period. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Battelle Toxicology Northwest Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines. Core study animals were weighed initially, and body weights and clinical findings were recorded on day 7 (female rats), day 8 (male rats and male and female mice), weekly thereafter, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1. Information on feed composition and contaminants is provided in Appendix G.

Animals were anesthetized with carbon dioxide, and blood was collected from the retroorbital plexus of clinical pathology rats on days 4 and 23 and from core study rats and mice at the end of the studies for hematology and clinical chemistry (rats only) analyses. Blood samples for hematology analyses were placed in tubes containing potassium EDTA. Packed cell volume; hemoglobin concentration; erythrocyte, platelet, and leukocyte counts; mean cell volume; mean cell hemoglobin; and mean cell hemoglobin concentration were determined using an Abbott Cell-Dyn 3700 Analyzer (Abbott Diagnostics Systems, Abbott Park, IL). Manual hematocrit values were determined using a microcentrifuge (Heraeus Haemofuge; Hanau, Germany) and a Damon/IEC capillary reader (International Equipment Co., Needham Heights, MA) for comparison to Cell-Dyn values for packed cell volume. Blood smears were stained with Romanowsky-type aqueous stain in a Wescor 1700 aerospray slide stainer (Wescor, Inc., Logan, UT). Leukocyte differential counts were based on classifying a minimum of 100 white cells. Reticulocytes were stained with new methylene blue and enumerated as a reticulocyte:erythrocyte ratio using the Miller disc method (Brecher and Schneiderman, 1950). Blood samples for clinical chemistry analyses were placed in tubes without anticoagulant and containing a separator gel, allowed to clot, and centrifuged. Parameters were determined using a Roche Hitachi 912 System (Roche Diagnostic Corporation, Indianapolis, IN). Table 1 lists the parameters measured.

At the end of the 3-month studies, samples were collected for sperm motility and vaginal cytology evaluations on rats and mice exposed to 0, 100, 200, or 400 ppm. The parameters evaluated are listed in Table 1. For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus,

estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed by the study laboratory pathologist on all chamber control and 400 ppm animals and 200 ppm female rats. In addition, the liver in the remaining groups of male rats, the kidney in the remaining groups of rats and mice, and the urinary bladder in the remaining groups of mice were examined. Table 1 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to a NTP Pathologists' Peer Review (PPR) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologists were resolved by the pathology peer review process. Final diagnoses for reviewed lesions represent a consensus of the PPR or a consensus between the study laboratory pathologist, NTP pathologist, QA pathologist(s), and the PPR coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

TABLE 1
Experimental Design and Materials and Methods in the Inhalation Studies of α-Pinene

2-Week Studies	3-Month Studies
Study Laboratory Battelle Toxicology Northwest (Richland, WA)	Battelle Toxicology Northwest (Richland, WA)
Strain and Species F344/N rats B6C3F1/N mice	F344/N rats B6C3F1/N mice
Animal Source Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies 11 days	Rats: 12 (males) or 13 (females) days Mice: 12 days
Average Age When Studies Began 6 weeks	Rats: 6 weeks Mice: 5 to 6 weeks
Date of First Exposure November 29, 2004	Rats: March 28 (males) or 29 (females), 2005 Mice: March 28, 2005
Duration of Exposure 6 hours plus T_{90} (12 minutes) per day, 5 days per week, for 16 (rats) or 17 (mice) days	6 hours plus T_{90} (10 minutes) per day, 5 days per week, for 14 weeks
Date of Last Exposure Rats: December 14, 2004 Mice: December 15, 2004	Rats: June 27 (males) or 28 (females), 2005 Mice: June 29 (males) or 30 (females), 2005
Necropsy Dates Rats: December 15, 2004 Mice: December 16, 2004	Rats: June 28 (males) or 29 (females), 2005 Mice: June 30 (males) or July 1 (females), 2005
Average Age at Necropsy 8 weeks	19 weeks
Size of Study Groups 5 males and 5 females	10 males and 10 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 2-week studies
Animals per Cage 1	1
Method of Animal Identification Tail tattoo	Same as 2-week studies
Diet NTP-2000 irradiated wafers (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum* (except during exposure periods); changed weekly	Same as 2-week studies

TABLE 1
Experimental Design and Materials and Methods in the Inhalation Studies of α-Pinene

2-Week Studies	3-Month Studies
Water Tap water (Richland, WA, municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI); available *ad libitum*	Same as 2-week studies
Cages Stainless steel, wire bottom (Lab Products, Inc., Seaford, DE); changed weekly	Same as 2-week studies; rotated weekly
Cageboard Untreated paper cage pan liner (Shepherd Specialty Papers, Kalamazoo, MI), changed daily	Same as 2-week studies
Chamber Air Supply Filters Single HEPA, changed annually; charcoal (RSE, Inc., New Baltimore, MI), new at study start; Purafil (Environmental Systems, Lynnwood, WA), new at study start	Same as 2-week studies
Chambers Stainless steel, excreta pan at each of six levels (Lab Products, Inc., Seaford, DE); chambers changed weekly; excreta pans changed daily	Same as 2-week studies
Chamber Environment Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Chamber air changes: 15 ± 2/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Chamber air changes: 15 ± 2/hour
Exposure Concentrations 0, 100, 200, 400, 800, and 1,600 ppm	0, 25, 50, 100, 200, and 400 ppm
Type and Frequency of Observation Observed twice daily; animals were weighed initially, on days 6 and 13, and at the end of the studies; clinical findings were recorded twice daily on exposure days and at the end of the studies.	Observed twice daily; core study animals were weighed initially, on day 7 (female rats), day 8 (male rats and male and female mice), weekly thereafter, and at the end of the studies; clinical findings were recorded on day 7 (female rats), day 8 (male rats and male and female mice), weekly thereafter, and at the end of the studies.
Method of Kill Carbon dioxide asphyxiation	Same as 2-week studies
Necropsy Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all core study animals. Organs weighed were heart, right kidney, liver, lung, spleen, right testis, and thymus.
Clinical Pathology None	Blood was collected from the retroorbital plexus of clinical pathology rats on days 4 and 23 and from core study animals at the end of the studies for hematology and clinical chemistry (rats only). ***Hematology***: hematocrit; packed cell volume; hemoglobin; erythrocyte, reticulocyte, and platelet counts; Howell-Jolly bodies (mice); mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte counts and differentials. ***Clinical chemistry***: urea nitrogen, creatinine, total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile salts

TABLE 1
Experimental Design and Materials and Methods in the Inhalation Studies of α-Pinene

2-Week Studies	3-Month Studies
Histopathology	
Histopathology was performed on 0, 400, 800, and 1,600 ppm rats and mice. In addition to gross lesions and tissue masses, the lung and nose were examined.	Complete histopathology was performed on 0 and 400 ppm core study rats and mice and 200 ppm female rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone, brain, clitoral gland, esophagus, eyes, gallbladder (mice), Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, larynx, liver, lung and mainstem bronchi, lymph nodes (mandibular, mesenteric, bronchial, mediastinal), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicles, thymus, thyroid gland, trachea, urinary bladder, and uterus. The liver of male rats, kidney of rats and mice, and urinary bladder of mice were examined in the remaining groups.
Sperm Motility and Vaginal Cytology	
None	At the end of the studies, sperm samples were collected from male animals in the 0, 100, 200, and 400 ppm groups for sperm motility evaluations. The following parameters were evaluated: sperm heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from females exposed to 0, 100, 200, or 400 ppm for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendix A as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the exposure-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis. Proportions of regular cycling females in each exposed group were compared to the control group using the Fisher exact test (Gart *et al.*, 1979). Tests for extended periods of estrus, diestrus, metestrus, and proestrus, as well as skipped estrus and skipped diestrus were constructed based on a Markov chain model proposed by Girard and Sager (1987). For each exposure group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within each stage as well as for skipping estrus or diestrus within a cycle. Equality of transition matrices among exposure groups and between the control group and each exposed group was tested using chi-square statistics.

QUALITY ASSURANCE METHODS

The 2-week and 3-month studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 3-month studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assessment contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Study Report.

GENETIC TOXICOLOGY

Bacterial Mutagenicity Test Protocol

Testing was performed using a modification of the protocol reported by Zeiger *et al*. (1992). α-Pinene was sent to the laboratory as a coded aliquot. It was incubated with the *Salmonella typhimurium* tester strains TA98 and TA100 and *Escherichia coli* tester strain WP2 *uvrA*/pKM101 (analogous to *S. typhimurium* strain TA102) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of α-pinene. The high dose was 10,000 μg/plate, which induced toxicity in some trials. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al*. (1990). At the end of the 3-month toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of five animals per exposure group. In addition, the percentage of polychromatic erythrocytes (PCEs) among a population of 1,000 erythrocytes was scored for each exposure group as a measure of bone marrow toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over exposure groups with a one-tail Cochran-Armitage trend test, followed by pairwise comparisons between each exposed group and the control group. In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single exposed group is less than or equal to 0.025 divided by the number of exposed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the

scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these samples and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this Toxicity Study Report represent a scientific judgment of the overall evidence for activity of the chemical in an assay.

RESULTS

RATS

2-WEEK STUDY

All rats exposed to 1,600 ppm were found dead by day 2 (Table 2). All rats exposed to 800 ppm were found dead by day 16. Final mean body weights of all exposed rats that survived to the end of the study were similar to those of the chamber controls; the mean body weight gain of 400 ppm females was significantly less than that of the chamber control group (Table 2). Abnormal breathing, ataxia, lethargy, and nasal/eye discharge occurred in one 1,600 ppm male. In rats exposed to 800 ppm, nasal/eye discharge was observed in two males and five females, ataxia was observed in two males and two females, and tremors and abnormal breathing were observed in one male. Nasal/eye discharge and tremors were observed in three females exposed to 400 ppm.

The absolute liver weights of 400 ppm males and 200 ppm females were significantly greater (21% and 19%, respectively) than those of the chamber controls (Table D1). The relative liver weights of 400 ppm males and all surviving groups of exposed females were significantly greater (up to 19%) than those of the chamber controls. The absolute kidney weight of 200 ppm females was significantly greater (14%) than that of the chamber controls, and the relative kidney weights of all surviving groups of exposed males and 200 and 400 ppm females were significantly greater (up to 16%) than those of the chamber controls. In females, the absolute lung weights of the 100 and 200 ppm groups and the relative lung weight of the 100 ppm group were significantly greater (up to 25%) than those of the chamber controls.

There were no exposure-related microscopic findings.

TABLE 2
Survival and Body Weights of Rats in the 2-Week Inhalation Study of α-Pinene[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)
Male					
0	5/5	101 ± 3	172 ± 3	71 ± 4	
100	5/5	101 ± 3	171 ± 2	70 ± 1	99
200	5/5	102 ± 3	173 ± 7	71 ± 5	100
400	5/5	100 ± 3	176 ± 4	75 ± 2	102
800	0/5[c]	101 ± 3	—	—	—
1,600	0/5[d]	102 ± 4	—	—	—
Female					
0	5/5	91 ± 2	125 ± 3	34 ± 2	
100	5/5	90 ± 2	130 ± 3	40 ± 2	104
200	5/5	91 ± 2	129 ± 2	38 ± 2	103
400	5/5	92 ± 1	118 ± 2	26 ± 2*	94
800	0/5[e]	92 ± 2	—	—	—
1,600	0/5[f]	91 ± 1	—	—	—

* Significantly different (P≤0.05) from the chamber control group by Dunnett's test
[a] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.
[b] Number of animals surviving at day 17/number initially in group
[c] Days of death: 8, 8, 8, 8, 16
[d] Days of death: 1, 1, 1, 1, 2
[e] Day of deaths: 8
[f] Day of deaths: 1

Exposure concentration selection rationale: Based on the mortality of 800 and 1,600 ppm rats and a lack of significant histopathologic findings in rats exposed to α-pinene at 400 ppm or less, exposure concentrations of 0, 25, 50, 100, 200, and 400 ppm α-pinene were selected for the 3-month rat study.

3-MONTH STUDY

All male rats survived to the end of the study (Table 3). Six 400 ppm female rats died during the study with no specific cause of death identified through gross examination or histopathologic analysis. The final mean body weights and mean body weight gains of females exposed to 400 ppm were significantly less than those of the chamber controls (Table 3; Figure 1); the final mean body weights and mean body weight gains of exposed males were similar to those of the chamber controls. No signs of toxicity (e.g., abnormal breathing or behavior) were noted during clinical observations.

TABLE 3
Survival and Body Weights of Rats in the 3-Month Inhalation Study of α-Pinene[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)
Male					
0	10/10	98 ± 3	335 ± 6	238 ± 5	
25	10/10	98 ± 2	329 ± 11	231 ± 9	98
50	10/10	98 ± 2	333 ± 6	235 ± 5	99
100	10/10	98 ± 2	334 ± 7	236 ± 5	100
200	10/10	96 ± 2	330 ± 4	234 ± 4	98
400	10/10	97 ± 2	322 ± 6	225 ± 7	96
Female					
0	10/10	89 ± 2	194 ± 3	105 ± 3	
25	10/10	89 ± 2	199 ± 4	110 ± 4	102
50	10/10	89 ± 2	206 ± 4	117 ± 4	106
100	10/10	88 ± 2	199 ± 3	112 ± 2	103
200	10/10	88 ± 2	201 ± 3	113 ± 2	104
400	4/10[c]	89 ± 2	159 ± 5**	72 ± 5**	82

** Significantly different (P≤0.01) from the chamber control group by Dunnett's test

[a] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

[b] Number of animals surviving at 14 weeks/number initially in group

[c] Weeks of death: 6, 6, 6, 6, 8, 13

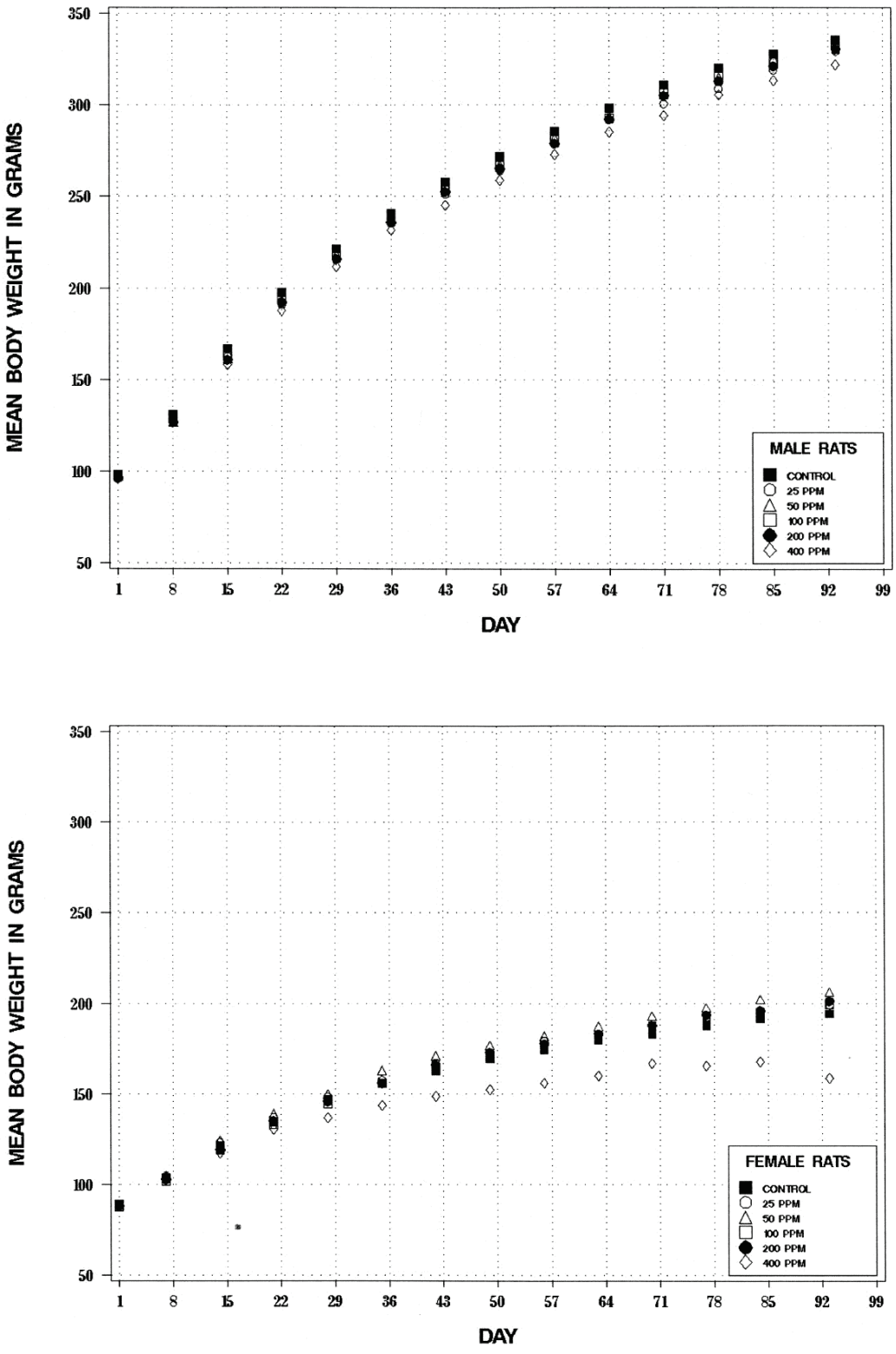

FIGURE 1
Growth Curves for Rats Exposed to α-Pinene by Inhalation for 3 Months

On day 4, there were mild exposure-related significant decreases in the leukocyte counts paired with mild significant decreases in the lymphocyte counts in 200 and 400 ppm male rats compared to concurrent controls (Table C1). These decreases ameliorated by day 23. At week 14, there were mild significant decreases in erythrocyte counts, hemoglobin concentrations, and hematocrit values in males exposed to 100 ppm or greater. The leukocyte changes likely represent a secondary treatment-associated stress effect. The exact mechanisms for the mild decreases in the erythron are not known. Alanine aminotransferase activities were significantly decreased in males and females exposed to 50 ppm or greater at week 14. Significantly decreased alanine aminotransferase activities were also seen in 400 ppm male rats on days 4 and 23. Significant decreases in alkaline phosphatase activities were observed in 400 ppm males and 200 and 400 ppm females on day 4 and in males and females exposed to 100 ppm or greater at week 14. The reason for the decreases in these enzyme activities is not known but may be related to alterations in liver metabolism or enzyme inhibition. The remaining significant differences in hematology and clinical chemistry parameters were not considered to be toxicologically relevant.

In males, the absolute liver weight of the 400 ppm group and the relative liver weights of groups exposed to 100 ppm or greater were significantly greater (up to 17%) than those of the chamber controls (Tables 4 and D2). In females, the absolute liver weights of the 50, 100, and 200 ppm groups and the relative liver weights of all exposed groups were significantly greater (up to 17%) than those of the chamber controls. The absolute heart weights of 100 and 200 ppm females and the relative heart weights of females exposed to 100 ppm or greater were significantly greater (up to 11%) than those of the chamber controls. The absolute kidney weights of male rats exposed to 100 ppm or greater and the relative kidney weights of males exposed to 50 ppm or greater were significantly greater (absolute: 11% to 25%; relative: 4% to 31%) than those of the chamber controls. In females, the absolute kidney weights of the 50 and 200 ppm groups and the relative kidney weights of the 200 and 400 ppm groups were significantly greater (up to 18%) than those of the chamber controls. The absolute and relative thymus weights of 400 ppm females and the relative thymus weight of 200 ppm females were significantly less than those of the chamber controls. The absolute and relative spleen weights of 400 ppm males were significantly greater than those of the chamber control group. The weight changes in lymphoid tissues were not accompanied by clinical chemistry or histopathologic changes indicative of immunotoxicity and, therefore, were not considered toxicologically relevant. With the exception of the male kidney, the organ weight changes in male and female rats were not accompanied by histopathologic lesions.

There were significantly decreased numbers of cauda sperm in 200 and 400 ppm males with 19% lower sperm per cauda in the 200 and 400 ppm groups compared to the chamber controls (Tables 5 and E1). Females in the 400 ppm group displayed an apparent increase in cycle length and a slight increase in the percentage of the cycle spent in metestrus, relative to the chamber control group (Table E2). However, the apparent increase in cycle length may be secondary to stress, as evidenced by lower body weight and mortality in the 400 ppm group. Alternatively, the apparent changes in the 400 ppm females may have been an artifact of having too few animals available to allow for meaningful interpretation. In addition, consideration of the complete cycle using a Markov analysis indicated that these exposed females did not spend significantly more time in any of the estrous stages than did the chamber control

group (Table E3). The minor changes in cycle length observed only in the exposure concentration group exhibiting overt toxicity, combined with a lack of ovarian histopathology findings, did not provide sufficient evidence for female reproductive toxicity potential under the conditions of the study. Based on these results, α-pinene exposure by inhalation exhibits the potential to be a reproductive toxicant in male rats, but not in female rats.

TABLE 4
Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
n	10	10	10	10	10	10
Necropsy body wt	335 ± 6	329 ± 11	333 ± 6	334 ± 7	330 ± 4	322 ± 6
R. Kidney						
Absolute	1.025 ± 0.019	1.012 ± 0.037	1.061 ± 0.026	1.137 ± 0.027**	1.209 ± 0.020**	1.286 ± 0.039**
Relative	3.058 ± 0.038	3.073 ± 0.037	3.186 ± 0.042*	3.405 ± 0.036**	3.660 ± 0.040**	3.991 ± 0.056**
Liver						
Absolute	10.54 ± 0.27	10.31 ± 0.40	10.44 ± 0.32	11.08 ± 0.36	11.37 ± 0.26	11.87 ± 0.45*
Relative	31.402 ± 0.375	31.270 ± 0.317	31.298 ± 0.490	33.152 ± 0.569*	34.393 ± 0.531**	36.807 ± 0.864**
Spleen						
Absolute	0.628 ± 0.012	0.630 ± 0.013	0.663 ± 0.014	0.659 ± 0.009	0.655 ± 0.010	0.677 ± 0.023*
Relative	1.874 ± 0.028	1.925 ± 0.045	1.997 ± 0.058	1.978 ± 0.030	1.983 ± 0.022	2.103 ± 0.057**
Female						
n	10	10	10	10	10	4
Necropsy body wt	194 ± 3	199 ± 4	206 ± 4	199 ± 3	201 ± 3	159 ± 5**
Heart						
Absolute	0.584 ± 0.010	0.612 ± 0.012	0.618 ± 0.010	0.629 ± 0.012*	0.638 ± 0.011**	0.530 ± 0.006*
Relative	3.010 ± 0.039	3.081 ± 0.054	3.002 ± 0.041	3.156 ± 0.034*	3.175 ± 0.049*	3.349 ± 0.084**
R. Kidney						
Absolute	0.618 ± 0.011	0.641 ± 0.009	0.680 ± 0.013**	0.659 ± 0.015	0.679 ± 0.014**	0.595 ± 0.021
Relative	3.185 ± 0.040	3.230 ± 0.062	3.301 ± 0.041	3.307 ± 0.058	3.376 ± 0.050*	3.757 ± 0.138**
Liver						
Absolute	5.486 ± 0.179	5.990 ± 0.121	6.270 ± 0.115**	6.269 ± 0.151**	6.424 ± 0.144**	4.840 ± 0.247
Relative	28.216 ± 0.637	30.152 ± 0.550**	30.438 ± 0.319**	31.459 ± 0.586**	31.916 ± 0.317**	30.470 ± 0.715**
Thymus						
Absolute	0.347 ± 0.012	0.349 ± 0.010	0.352 ± 0.010	0.346 ± 0.010	0.330 ± 0.014	0.204 ± 0.010**
Relative	1.785 ± 0.054	1.751 ± 0.029	1.707 ± 0.041	1.739 ± 0.048	1.638 ± 0.058*	1.286 ± 0.035**

* Significantly different (P≤0.05) from the chamber control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE 5
Epididymal Spermatozoal Measurements for Male Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
n	10	10	9	10
Epididymal spermatozoal measurements				
Sperm motility (%)	91.73 ± 1.26	91.40 ± 0.93	91.24 ± 0.80	90.93 ± 0.89
Sperm (10^3/mg cauda epididymis)	615.0 ± 34.3	596.5 ± 31.8	526.3 ± 19.0	547.4 ± 14.0
Sperm (10^6/cauda epididymis)	120.89 ± 6.79	113.16 ± 3.11	97.52 ± 3.51**	98.40 ± 3.02**

** Significantly different (P≤0.01) from the chamber control group by Shirley's test.
[a] Data are presented as mean ± standard error.

Renal tubule lesions including granular casts, hyaline droplets, and nephropathy were observed in male rats exposed to α-pinene (Tables 6 and A1). In all male groups exposed to α-pinene, there were significantly increased incidences of granular casts and hyaline droplet accumulation and the severities of these lesions increased with increasing exposure concentration. Granular casts were not observed in the chamber controls (Plates 1 and 2), and in exposed groups the mean severity ranged from minimal in males exposed to 25 ppm to moderate in males exposed to 400 ppm. Casts occurred in the lumens of the tubules along the outer medulla and were composed of pale, eosinophilic, granular cell debris that filled and dilated the tubular lumens (Plates 3 and 4).

Hyaline droplet accumulation occurred in the cytoplasm of the renal proximal convoluted tubule epithelial cells, and the severity ranged from minimal in males exposed to 25 ppm to moderate in males exposed to 400 ppm (Tables 6 and A1). In the chamber controls, the droplets occurred as individual, uniformly fine, round eosinophilic globules in the epithelial cells of clusters of tubules (Plates 5 and 6). Intervening areas of tubules devoid of cytoplasmic droplets separated these clusters of tubules. As the exposure concentration and severity increased, the droplets were increasingly larger and varied from round to rectangular and often occurred in clumps (Plates 7 and 8). At the highest severity grade, the accumulations varied from small to large irregular globular to rectangular to crystalline, refractile forms. Although evident in the H&E-stained sections, when stained with the Mallory-Heidenhain method for visualization of protein, accumulations were more prominent and allowed better quantification and characterization of the droplets.

With the exception of one chamber control rat, nephropathy occurred in all males, with the mean severity increasing from minimal in chamber control males to moderate in males exposed to 400 ppm (Tables 6 and A1). Nephropathy was characterized by multifocal clusters of regenerating tubules surrounded by thickening of tubular basement membranes, individual tubular epithelial cell necrosis, widely scattered protein casts, and associated infiltrates of lymphocytes within the interstitium.

There were no exposure-related microscopic findings in females, including those that died before the end of the study.

TABLE 6
Incidences of Nonneoplastic Lesions of the Kidney in Male Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Number Examined Microscopically	10	10	10	10	10	10
Casts, Granular[a]	0	9** (1.0)[b]	10** (1.2)	10** (1.5)	10** (2.5)	10** (3.0)
Accumulation, Hyaline Droplet	1 (2.0)	10** (1.1)	10** (1.8)	10** (2.0)	10** (2.7)	10** (3.0)
Nephropathy	9 (1.1)	10 (1.6)	10 (2.0)	10 (2.0)	10 (2.5)	10 (3.0)

** Significantly different (P≤0.01) from the chamber control group by the Fisher exact test

[a] Number of animals with lesion

[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

MICE

2-WEEK STUDY

All 800 and 1,600 ppm males and females died early (Table 7). The final mean body weights and mean body weight gains of all surviving groups of exposed mice were similar to those of the chamber controls. Lethargy and abnormal breathing were observed in three 800 ppm males and two 1,600 ppm males, and lethargy was observed in one 1,600 ppm female. Ataxia was observed in two 800 ppm males, two 1,600 ppm males, and four 800 ppm females.

TABLE 7
Survival and Body Weights of Mice in the 2-Week Inhalation Study of α-Pinene[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)
Male					
0	5/5	23.7 ± 0.4	27.9 ± 0.7	4.1 ± 0.4	
100	5/5	23.4 ± 0.7	26.7 ± 0.7	3.4 ± 0.3	96
200	5/5	23.1 ± 0.8	26.6 ± 0.9	3.5 ± 0.4	95
400	5/5	23.9 ± 0.5	27.0 ± 0.6	3.1 ± 0.2	97
800	0/5[c]	23.5 ± 0.5	—	—	—
1,600	0/5[d]	23.5 ± 0.6	—	—	—
Female					
0	5/5	20.2 ± 0.4	23.0 ± 0.4	2.8 ± 0.3	
100	5/5	20.6 ± 0.5	23.6 ± 0.5	3.0 ± 0.3	103
200	5/5	20.2 ± 0.5	23.2 ± 0.7	3.0 ± 0.7	101
400	5/5	19.9 ± 0.5	22.6 ± 0.5	2.7 ± 0.3	99
800	0/5[e]	19.8 ± 0.3	—	—	—
1,600	0/5[f]	19.8 ± 0.2	—	—	—

[a] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.
[b] Number of animals surviving at day 18/number initially in group
[c] Days of death: 2, 2, 3, 4, 16
[d] Days of death: 1, 1, 1, 1, 2
[e] Day of deaths: 2
[f] Day of deaths: 1

The absolute and relative liver weights of 400 ppm males and females and the relative liver weight of 200 ppm males were significantly greater (up to 20%) than those of the chamber controls (Table D3). The absolute and relative kidney weights of 100 ppm females were significantly greater (18% and 15%, respectively) than those of the chamber controls as was the relative kidney weight of 400 ppm males (12%).

In the nose, there were significantly increased incidences of minimal olfactory epithelial degeneration in 800 and 1,600 ppm males (chamber controls, 0/5; 100 ppm, 0/0; 200 ppm, 0/0; 400 ppm, 0/5; 800 ppm, 5/5; 1,600 ppm, 4/5) and females (0/5, 0/0, 0/0, 0/5, 4/5, 5/5). Degeneration was characterized by slight disorganization of the normal olfactory architecture in the Level I and II nasal sections and the epithelial cells in the mid layers of the olfactory epithelium were often pyknotic. The mucosa often had an undulating appearance, and strands of proteinaceous debris and/or mucus were present in the nasal passages.

Exposure concentration selection rationale: Based on decreased survival and nonneoplastic lesions in the nose of 800 and 1,600 ppm males and females observed in this study, exposure concentrations of 0, 25, 50, 100, 200, and 400 ppm α-pinene were selected for the 3-month study in mice.

3-MONTH STUDY

All mice survived to the end of the study (Table 8). The final mean body weights and body weight gains of exposed males and females were similar to those of the chamber controls (Table 8; Figure 2). No clinical findings related to α-pinene exposure were observed.

TABLE 8
Survival and Body Weights of Mice in the 3-Month Inhalation Study of α-Pinene[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)
Male					
0	10/10	22.9 ± 0.2	37.1 ± 0.6	14.3 ± 0.6	
25	10/10	23.0 ± 0.3	36.9 ± 0.7	13.9 ± 0.8	99
50	10/10	22.7 ± 0.3	38.3 ± 0.9	15.6 ± 0.8	103
100	10/10	22.5 ± 0.2	35.9 ± 0.7	13.4 ± 0.7	97
200	10/10	22.8 ± 0.3	35.5 ± 1.0	12.7 ± 0.9	96
400	10/10	22.8 ± 0.2	36.2 ± 0.5	13.5 ± 0.4	98
Female					
0	10/10	19.5 ± 0.4	31.5 ± 0.6	12.0 ± 0.5	
25	10/10	19.6 ± 0.4	30.3 ± 0.6	10.8 ± 0.7	96
50	10/10	19.7 ± 0.3	32.7 ± 0.7	12.9 ± 0.7	104
100	10/10	19.7 ± 0.4	31.5 ± 1.1	11.8 ± 0.9	100
200	10/10	19.3 ± 0.3	30.7 ± 0.6	11.4 ± 0.6	97
400	10/10	19.4 ± 0.3	30.6 ± 0.5	11.2 ± 0.4	97

[a] Weights and weight changes are given as mean ± standard error.
[b] Number of animals surviving at 14 weeks/number initially in group

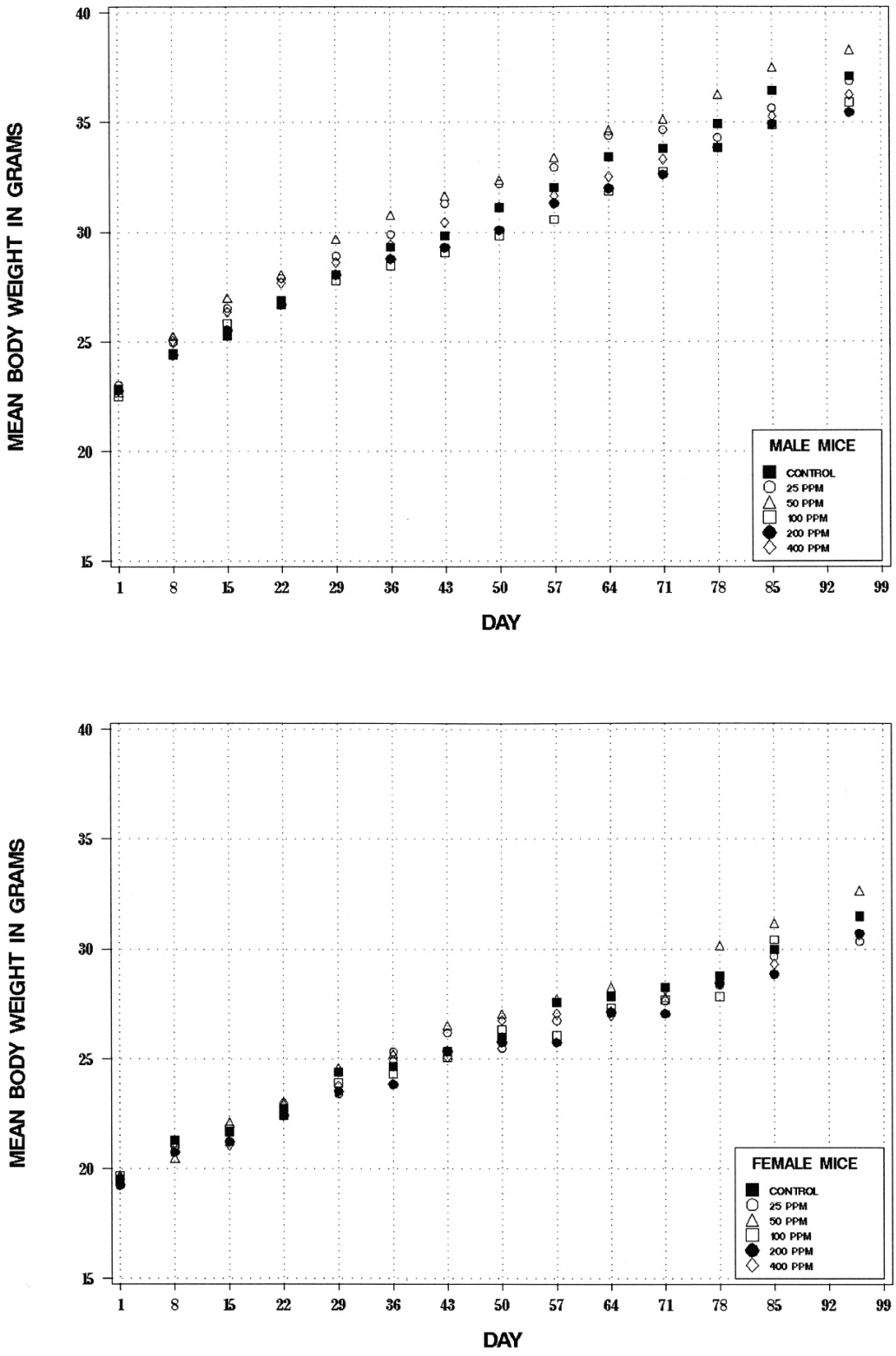

FIGURE 2
Growth Curves for Mice Exposed to α-Pinene by Inhalation for 3 Months

At the end of the study, there were small but statistically significant decreases in erythrocyte counts in 200 and 400 ppm females and in the hemoglobin concentration and the hematocrit value in 400 ppm females compared to concurrent controls (Table C2). Decreases in erythrocyte count and hematocrit value also occurred in 400 ppm males. Leukocyte and lymphocyte counts were significantly decreased in 400 ppm males. The leukocyte changes likely represent a secondary treatment-associated stress effect. The exact mechanism for the mild decreases in the erythron are not known. Other significant changes in hematology parameters were not toxicologically relevant.

The absolute liver weights of 400 ppm males and females and the relative liver weights of 200 and 400 ppm males and 100, 200, and 400 ppm females were significantly greater (up to 24%) than those of the chamber controls (Tables 9 and D4). The absolute and relative thymus weights of 400 ppm males were significantly less than those of the chamber controls. The absolute kidney weights of 200 and 400 ppm males were significantly less than those of the chamber controls (11% and 7%, respectively). These organ weight changes were not accompanied by histopathologic lesions.

TABLE 9
Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
n	10	10	10	10	10	10
Male						
Necropsy body wt	37.1 ± 0.6	36.9 ± 0.7	38.3 ± 0.9	35.9 ± 0.7	35.5 ± 1.0	36.2 ± 0.5
R. Kidney						
Absolute	0.330 ± 0.006	0.318 ± 0.009	0.336 ± 0.010	0.309 ± 0.008	0.295 ± 0.006*	0.307 ± 0.007*
Relative	8.903 ± 0.167	8.629 ± 0.208	8.793 ± 0.267	8.617 ± 0.205	8.348 ± 0.145	8.469 ± 0.155
Liver						
Absolute	1.617 ± 0.022	1.589 ± 0.028	1.702 ± 0.040	1.637 ± 0.024	1.660 ± 0.043	1.957 ± 0.057**
Relative	43.671 ± 0.880	43.123 ± 0.458	44.487 ± 0.806	45.651 ± 0.678	46.903 ± 0.750*	54.009 ± 1.465**
Thymus						
Absolute	0.066 ± 0.004	0.063 ± 0.004	0.067 ± 0.003	0.057 ± 0.001	0.062 ± 0.004	0.051 ± 0.003**
Relative	1.777 ± 0.081	1.699 ± 0.090	1.742 ± 0.063	1.591 ± 0.052	1.739 ± 0.115	1.397 ± 0.081**
Female						
Necropsy body wt	31.5 ± 0.6	30.3 ± 0.6	32.7 ± 0.7	31.5 ± 1.1	30.7 ± 0.6	30.6 ± 0.5
Liver						
Absolute	1.466 ± 0.041	1.475 ± 0.053	1.442 ± 0.036	1.548 ± 0.053	1.587 ± 0.037	1.730 ± 0.032**
Relative	46.542 ± 0.988	48.567 ± 1.239	44.214 ± 0.880	49.280 ± 0.672*	51.728 ± 0.795**	56.511 ± 0.705**

* Significantly different (P≤0.05) from the chamber control group by Williams' test
** Significantly different (P≤0.01) from the chamber control group by Williams' or Dunnett's test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

There were significantly decreased numbers of sperm per mg cauda in 200 and 400 ppm males (24% and 37%, respectively) and cauda sperm in 100, 200, and 400 ppm males (25%, 33%, and 40%, respectively; Tables 10 and E4). There were no changes in the proportion of regularly cycling females, estrous cycle length, or percentage of time spent in the individual stages of the estrous cycle of female mice at any exposure concentration (Table E5) and there were no ovarian histopathologic findings. Therefore, α-pinene exposure via inhalation exhibits the potential to be a reproductive toxicant in male mice, but not in female mice.

In the urinary bladder, there were significantly increased incidences of transitional epithelium hyperplasia in males and females exposed to 100 ppm or greater (Tables 11, A3, and A4). The incidences and the severities of this lesion increased in an exposure concentration-related manner. This lesion was characterized by a relatively uniform increase in mucosal thickness with an increase in the size of the transitional epithelial cells, and the number of epithelial cell layers from two or three in controls (Plates 9 and 10) to four or more layers in affected mice (Plates 11 and 12). The hyperplastic epithelium had cells of more uniform size, with loss of the superficial large cells and a less distinct basal cell layer. Cells frequently exhibited increased amounts of dark eosinophilic to slightly basophilic cytoplasm with enlarged nuclei and occasional mitotic figures. Individually necrotic transitional epithelial cells were sometimes scattered along the luminal surface.

TABLE 10
Epididymal Spermatozoal Measurements of Male Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
n	10	10	10	10
Epididymal spermatozoal measurements				
Sperm motility (%)	90.25 ± 0.34	88.31 ± 0.86	89.74 ± 0.80	87.95 ± 1.08
Sperm (10^3/mg cauda epididymis)	704.8 ± 64.9	690.7 ± 55.9	537.5 ± 27.0*	445.8 ± 13.5**
Sperm (10^6/cauda epididymis)	24.45 ± 0.95	18.40 ± 0.41**	16.48 ± 0.72**	14.64 ± 0.25**

* Significantly different (P≤0.05) from the chamber control group by Shirley's test.
** P≤0.01
[a] Data are presented as mean ± standard error.

TABLE 11
Incidences of Nonneoplastic Lesions of the Urinary Bladder in Mice in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
Number Examined Microscopically	10	10	10	10	10	10
Transitional Epithelium, Hyperplasia[a]	0	0	0	7** (1.0)[b]	10** (2.0)	10** (2.5)
Female						
Number Examined Microscopically	10	10	10	10	10	10
Transitional Epithelium, Hyperplasia	0	0	0	6** (1.0)	10** (1.6)	10** (2.2)

** Significantly different ($P \leq 0.01$) from the chamber control group by the Fisher exact test
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

GENETIC TOXICOLOGY

α-Pinene (5 to 10,000 µg/plate) was not mutagenic in *Salmonella typhimurium* strains TA98 or TA100 or in *Escherichia coli* strain WP2 *uvrA*/pKM101 with or without rat liver S9 activation enzymes (Table B1).

No increases in the frequencies of micronucleated erythrocytes (biomarkers of chromosomal damage) were observed in peripheral blood of male or female mice in the 3-month inhalation study (Table B2). In addition, no significant changes in the percentages of polychromatic erythrocytes (immature erythrocytes) were noted in either male or female mice exposed to α-pinene, indicating an absence of bone marrow toxicity.

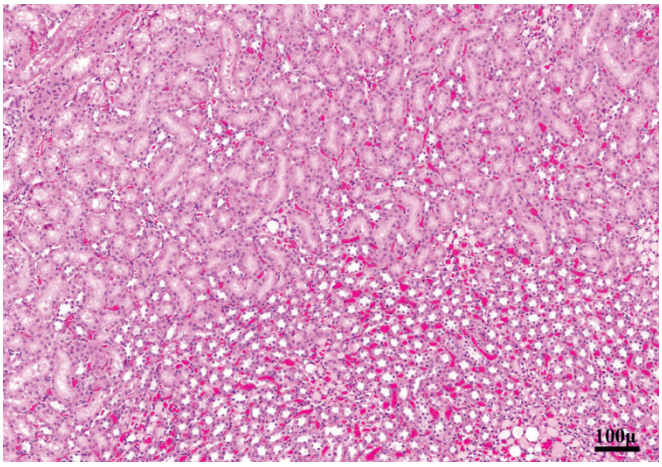

PLATE 1
Kidney of a chamber control male F344/N rat in the 3-month inhalation study of α-pinene. H&E

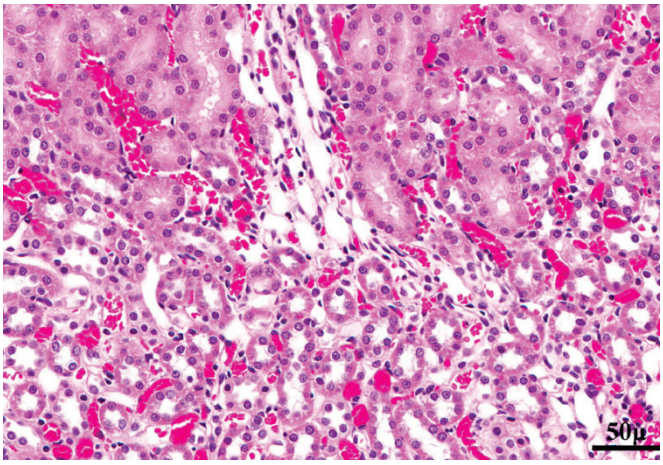

PLATE 2
Higher magnification of Plate 1. H&E

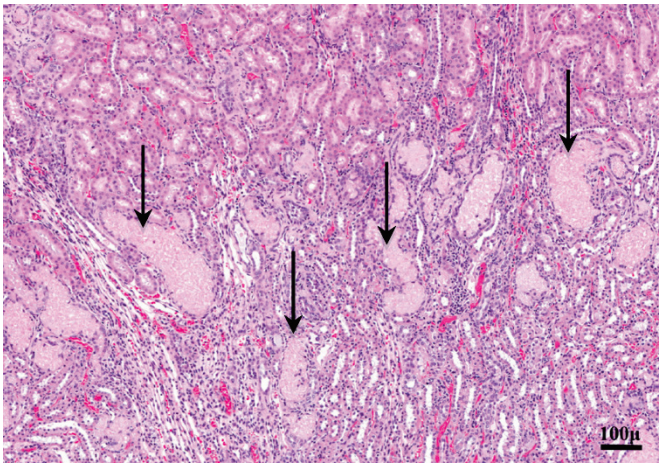

PLATE 3
Kidney of a male F344/N rat exposed to 400 ppm α-pinene by inhalation for 3 months. Note the numerous tubules (arrows) containing granular casts, which are considered a hallmark of α2µ-globulin nephropathy in male rats. H&E

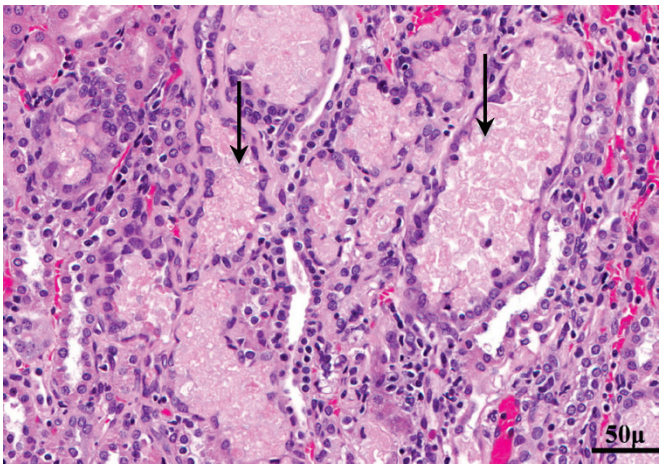

PLATE 4
Higher magnification of Plate 3. H&E

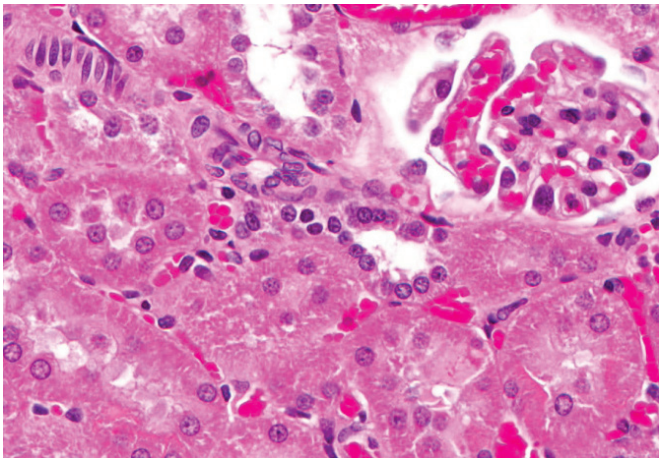

PLATE 5
Kidney of a chamber control male F344/N rat in the 3-month inhalation study of α-pinene. H&E

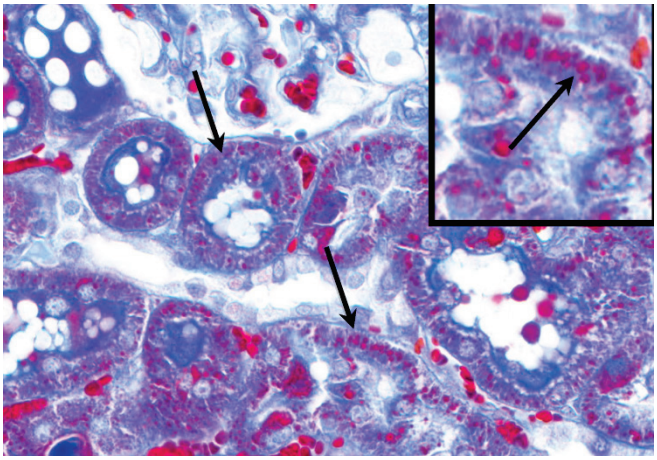

PLATE 6
Kidney of a chamber control male F344/N rat in the 3-month inhalation study of α-pinene. Note the presence of small protein droplets (arrows) that are normally present in the renal tubule epithelium of male rats. The inset highlights the protein droplets. Mallory-Heidenhain

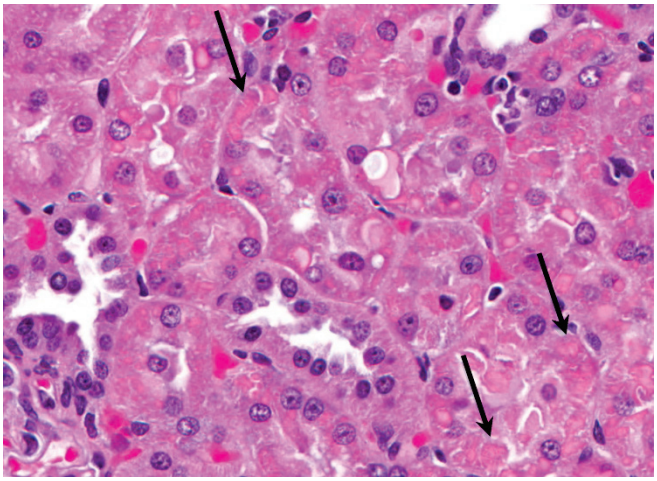

PLATE 7
α2μ-Globulin nephropathy in the kidney of a male F344/N rat exposed to 400 ppm α-pinene by inhalation for 3 months. Note the accumulation of large, irregular, coalescing protein droplets (arrows) in the renal tubule epithelium. H&E

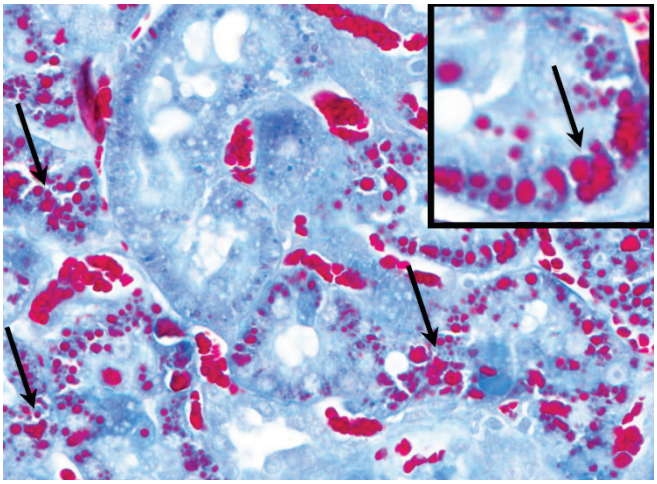

PLATE 8
α2μ-Globulin nephropathy in the kidney of a male F344/N rat exposed to 400 ppm α-pinene by inhalation for 3 months. Note the accumulation of large, irregular, coalescing protein droplets (arrows) in the renal tubule epithelium. The inset highlights the large irregular, coalescing protein droplets. Mallory-Heidenhain

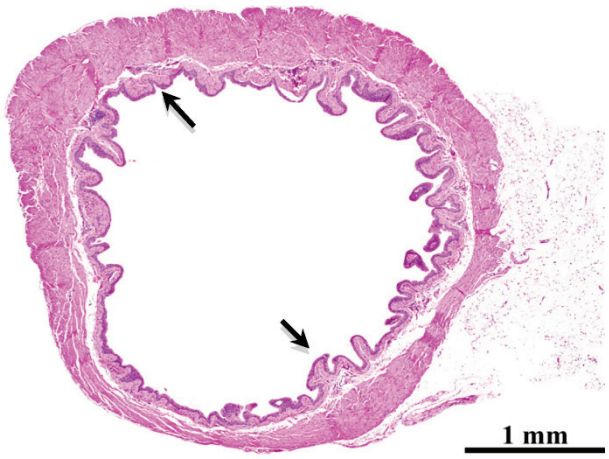

PLATE 9
Urinary bladder of a chamber control female B6C3F1/N mouse in the 3-month inhalation study of α-pinene. Note the normal transitional epithelium (arrows). H&E

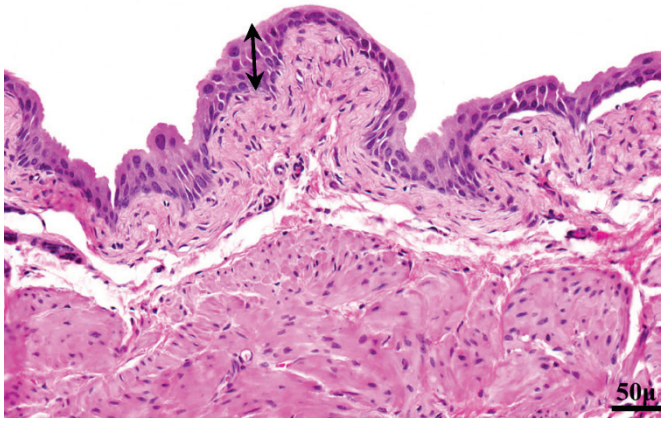

PLATE 10
Higher magnification of Plate 9 showing normal transitional epithelium (arrow). H&E

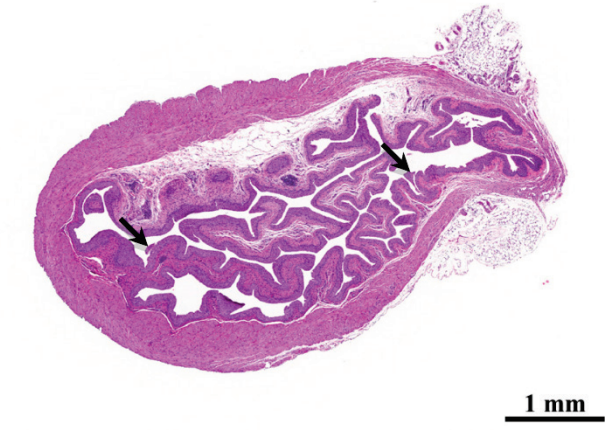

PLATE 11
Urinary bladder of a female B6C3F1/N mouse exposed to 400 ppm α-pinene by inhalation for 3 months. Note the markedly hyperplastic transitional epithelium (arrows). H&E

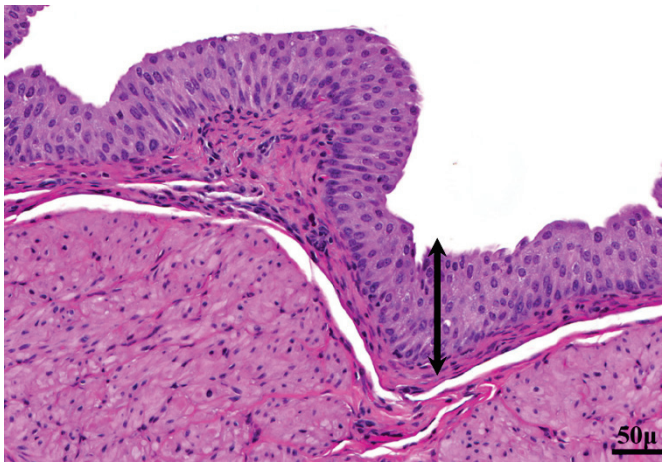

PLATE 12
Higher magnification of Plate 11 showing the hyperplastic transitional epithelium (arrow). H&E

DISCUSSION

α-Pinene is the primary constituent of turpentine: an oleoresin extracted from coniferous trees, especially of the genus *Pinus*. α-Pinene belongs to a class of chemicals known as monoterpenes, which contain two isoprene units and include other members that are also present in turpentine: β-pinene, Δ^3-carene, and *d*-limonene. In addition to its presence in turpentine, which is used primarily as a solvent, α-pinene is used as a flavor enhancer in foods or as a fragrance ingredient in personal care products, household cleaners, or air fresheners. Exposure to α-pinene can occur occupationally (e.g., wood processing industry, painting) (Demers *et al*., 2000; Rosenberg *et al*., 2002) or through the use of household goods (Rastogi *et al.*, 2001). Workplace exposure limits for α-pinene include a legal permissible exposure limit (PEL) and recommended airborne exposure limit (REL) of 100 ppm (as turpentine) (NIOSH, 2010) and a threshold limit value (TLV) of 20 ppm averaged over an 8-hour workshift (ACGIH, 2015). Despite widespread exposure potential, there are very little data available for characterizing the toxicity or carcinogenicity of this compound. The present report describes the 2-week and 3-month toxicity studies of α-pinene administered to F344/N rats and B6C3F1/N mice by inhalation. The toxicity targets of α-pinene were generally consistent across species and included the liver, urinary system (kidneys in rats and bladder in mice), and cauda epididymal sperm.

Exposure concentrations for the 2-week studies with α-pinene were selected based on early studies with turpentine in which rats were exposed in a chamber to concentrations of turpentine roughly estimated at 5,000 to 10,000 mg/m^3 or 897 to 1,795 ppm (Chapman, 1941). In the 2-week studies with α-pinene, the two highest exposure concentrations (800 and 1,600 ppm) were overtly toxic to both rats and mice, resulting in clinical findings of toxicity (e.g., ataxia, tremors, abnormal breathing) and death. In the remaining exposed groups, there was evidence of a general increase in absolute and/or relative liver and kidney weights compared to chamber control rats and mice of both sexes. The greatest weight changes (approximately 17% increases in relative liver weight in the 400 ppm male rats and mice) were not considered to be life threatening. Therefore, 400 ppm was selected as the high concentration for the 3-month studies in rats and mice.

In the 3-month studies, female rats appeared to be more sensitive to α-pinene than male rats or male or female mice. The high exposure concentration of 400 ppm resulted in the death of 60% of female rats and lower body weights in surviving females compared to the chamber controls but was not overtly toxic to male rats, male mice, or female mice. Additionally, an exposure concentration-dependent increase in relative heart weight at 100 ppm or greater was observed only in female rats; however, there were no accompanying histopathologic lesions in the heart.

While there are no data characterizing the effects of α-pinene on the urinary system of rodents or humans, the association between exposure to turpentine and acute renal injury in humans has long been recognized (Chapman, 1941). The Occupational Safety and Health Administration (2013) lists toxic glomerulonephritis and bladder irritation with hematuria, albuminuria, oliguria, and dysuria among human effects associated with overexposure to turpentine vapors. However, the chronic effects of turpentine, or α-pinene, to the urinary system remain unknown. In the only identified study on the subject, Chapman (1941) exposed rats to turpentine vapors at exposure concentrations roughly estimated by the National Toxicology Program (NTP) at 5,000 to 10,000 mg/m^3 or 897 to 1,795 ppm for periods ranging from 6.5 hours on a single day to 293 hours over the course of a year and did not find any histopathologic signs of renal injury.

In the current studies, there were multiple indications of urinary system injury following α-pinene exposure. In the 2-week and 3-month studies, relative kidney weights were increased in an exposure concentration-dependent manner in male and female rats. In male rats, exposure to α-pinene was associated with increases in the incidence and severity of hyaline droplet accumulation within the epithelial cells of the P2 segment of the renal tubule. In addition, prominent granular casts were observed in the lumens of the renal tubules along the corticomedullary junction. These casts are an indication of previous injury and death of the renal tubule epithelium with accumulation of the cellular debris (casts) in the tubules. There was also evidence of exacerbation of the chronic progressive nephropathy that is a common spontaneous change in the kidneys of male rats as evidenced by an exposure concentration-related increase in the severity of this lesion. Nephropathy was characterized by randomly distributed multifocal clusters of regenerating tubules within the parenchyma of the kidney. The presence of these nonneoplastic lesions in the kidney is suggestive of α2μ-globulin nephropathy, a renal syndrome that occurs in male but not female F334/N rats and that has been linked to the development of renal tubule neoplasms (Swenberg, 1993). This syndrome has been produced by structurally diverse chemicals and is thought to be secondary to toxicity caused by accumulation of hyaline droplets within the renal tubule epithelial cells. The kidney lesions observed in the current study are consistent with those observed in 90-day studies of *d*-limonene, decalin, and propylene glycol mono-*t*-butyl ether for which the mechanism of renal tumor induction in 2-year studies was considered to be related to α2μ-globulin nephropathy (Doi *et al*., 2007). The lesions meet some of the criteria used by the United States Environmental Protection Agency (1991) and the International Agency for Research on Cancer (1999) for induction of renal tumors by this mechanism. However, it should be noted that measures of α2μ-globulin and cell proliferation, which are also criteria used by these agencies, were not performed in the current studies. While it is possible that the observed kidney lesions are secondary to α2μ-globulin nephropathy, the increases in kidney weights in both male and female rats suggest that another independent mechanism of toxicity may have played a role in the lesion development.

Further evidence of α-pinene targeting the urinary system was found in the 3-month study of male and female mice. The primary effect in mice caused by exposure to α-pinene was an increased incidence of transitional epithelium hyperplasia of the urinary bladder in males and females exposed to 100 ppm or more, the severity of which increased with increasing exposure concentration. This finding is relatively rare among subchronic mouse studies at the NTP,

with few test articles identified (e.g., 2,2-bis(bromomethyl)-1,3-propanediol, methyl ethyl ketoxime, *t*-butyl alcohol) that elicited treatment-dependent increased incidences of urinary bladder transitional epithelium hyperplasia in mice following 13 weeks of exposure (NTP, 1995, 1996, 1999). Transitional epithelium hyperplasia in the urinary bladder can be either reparative (e.g., regenerative or reactive) or preneoplastic, but there are no histologic features that can be used to reliably distinguish between the two etiologies (Koss and Hoda, 2012). Reparative hyperplasia is a common secondary response to inflammation and/or necrosis in the urinary bladder and may also occur when urinary calculi (solid particles or "stones") are present. Preneoplastic hyperplasia of the transitional epithelium is considered a component lesion in the continuum to neoplasia in the urinary bladder, and when present, cellular atypia or atypical growth patterns may provide plausible evidence that the hyperplasia is preneoplastic. Specific histopathologic indicators of either type of hyperplasia (e.g., calculi for reparative, cellular atypia for preneoplastic) were not evident in male or female mice from the current study; therefore, the neoplastic potential of the transitional epithelium hyperplasia of the urinary bladder that did occur is uncertain. Importantly, hyperplasia is often noted in studies of urinary bladder carcinogens in mice. In one class of urinary bladder carcinogens, irritation from chemically induced foreign bodies in the urinary bladder leads to reparative hyperplasia and eventually cancer, exemplified by uracil (Sakata *et al*., 1988), which is a nongenotoxic urinary bladder carcinogen with promoting potential (Shirai *et al*., 1987; Fukushima *et al*., 1992). A second class of urinary bladder carcinogens in mice, including *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (Ogawa *et al*., 1998) elicit DNA damage, and induce preneoplastic hyperplasia followed by tumor production. Therefore, it is plausible that a chemical or compound that is carcinogenic to the transitional epithelium of the urinary bladder is likely to cause an increased incidence of transitional epithelium hyperplasia as a precursor to neoplasia.

In addition to the urinary system, the male reproductive system appeared to be a target of α-pinene toxicity, with more pronounced effects in mice than in rats. In male rats, absolute sperm per cauda decreased by approximately 20% at the two highest exposure concentrations compared to chamber controls. There was an accompanying minor decrease in epididymal weights that did not reach significance. Therefore, the possibility that the change in absolute sperm per cauda was due to a decrease in epididymal weight cannot be ruled out. In male mice, sperm per mg cauda decreased by 24% and 37% in the 200 and 400 ppm groups, respectively. Although histopathologic changes in the epididymides or testes would be expected to accompany decreases of this magnitude, artifacts in the male reproductive tract tissues resulting from formalin fixation precluded a definitive assessment of those tissues (Liang *et al*., 2000; Latendresse *et al*., 2002). Since the α-pinene studies were conducted, the NTP has implemented the use of Davidson's fluid for fixation of male reproductive tissue, which has greatly improved resolution. Further studies on the effects of α-pinene on reproductive function are warranted. Under the conditions of the 3-month studies in rats and mice, there was no evidence of female reproductive toxicity.

Finally, α-pinene elicited relatively weak signals of potential toxicity in the liver of mice and rats. Liver weights were increased in exposed animals of both sexes and species. In female rats, a significant increase in relative liver weight was observed at the lowest exposure tested (25 ppm). Increased liver weight is a common finding in toxicity studies

and can be associated with induction of liver metabolizing enzymes. α-Pinene has been shown to increase both phase I and phase II metabolizing enzymes *in vitro* and *in vivo* (Pap and Szarvas, 1976; Austin *et al*., 1988; Hiroi *et al*., 1995; Lamb *et al*., 2004).

There is limited overlap in target sites across monoterpene class members that have been tested by the NTP, including β-myrcene (NTP, 2010), *d*-limonene (NTP, 1990), citral (NTP, 2003), geranyl acetate (NTP, 1987) and α,β-thujone (NTP, 2011). α-Pinene, citral, and *d*-limonene elicited kidney lesions in male rats suggestive of an α2μ-globulin mechanism of toxicity (e.g., granular casts and accumulation of hyaline droplets); however, there does not appear to be a typical monoterpene toxicity profile. For example, α-pinene was the only monoterpene tested that induced hyperplasia of the urinary bladder in mice or decreased cauda epididymal sperm in male mice and rats, while many of the other monoterpenes did not elicit specific histopathologic changes in mice in the 3-month studies, but had unique targets in male and female rats, including the nose (β-myrcene), forestomach and bone marrow (citral), and brain (α,β-thujone).

In studies performed by the NTP, α-pinene was not mutagenic *in vitro* or *in vivo*. These results are consistent with the majority of previous assessments of genotoxicity of α-pinene (Florin *et al*., 1980; Connor *et al*., 1985; Gomes-Carneiro *et al*., 2005; Gminski *et al*., 2010). Additionally, many other structurally related monoterpenes have been found to be nongenotoxic, regardless of their carcinogenicity *in vivo* (NTP, 1987, 1990, 2003, 2010, 2011). In contrast, increased frequencies of chromosomal aberrations and micronuclei (some of which contained kinetochores) were observed in V79 Chinese hamster cells exposed to α-pinene (Catanzaro *et al*., 2012). These clastogenic and aneugenic effects were accompanied by increased formation of reactive oxygen species and disruption of the mitotic spindle (Catanzaro *et al*., 2012). The *in vivo* micronucleus test is only sensitive to test articles (or their reactive metabolites) that reach the bone marrow. In the micronucleus studies conducted by NTP (Table B2), α-pinene did not alter the percentage of polychromatic erythrocytes in peripheral blood of mice and did not affect the hematopoietic system in rats or mice (Tables A1 through A4). These observations suggest that α-pinene either was not toxic to bone marrow or did not reach the bone marrow compartment. Given the findings by Catanzaro *et al*. (2012) and the observation that monoterpenes structurally related to α-pinene are carcinogenic, additional testing using the comet assay to assess the potential for α-pinene to induce DNA damage in cells of the lung (site of contact), liver, kidney, or urinary bladder (sites of lesions in the 3-month studies) may be informative.

Under the conditions of the 3-month inhalation studies, there were treatment-related lesions in male and female rats and mice. The major targets from α-pinene exposure in rats and mice included the liver, urinary system (kidney of rats and urinary bladder of mice), and cauda epididymal sperm. The most sensitive measures of α-pinene exposure in each species and sex were increased incidences of kidney lesions in male rats [lowest-observed-effect level (LOEL)=25 ppm], increased relative liver weights in female rats (LOEL=25 ppm) without accompanying histopathologic changes, decreased sperm per cauda and increased incidences of transitional epithelium hyperplasia

of the urinary bladder in male mice (LOEL=100 ppm), and increased incidences of transitional epithelium hyperplasia of the urinary bladder in female mice (LOEL=100 ppm).

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APPENDIX A
SUMMARY OF NEOPLASMS AND NONNEOPLASTIC LESIONS IN RATS AND MICE

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal kill	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Clear cell focus						1 (10%)
Hepatodiaphragmatic nodule		1 (10%)				1 (10%)
Mesentery	(0)	(1)	(0)	(0)	(0)	(0)
Fat, necrosis		1 (100%)				
Stomach, glandular	(10)	(0)	(0)	(0)	(0)	(10)
Mineralization						1 (10%)
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(0)	(10)
Cardiomyopathy	6 (60%)					8 (80%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Epididymis	(10)	(0)	(0)	(0)	(0)	(10)
Granuloma sperm						1 (10%)
Hematopoietic System						
Lymph node, mediastinal	(7)	(0)	(0)	(0)	(0)	(4)
Infiltration cellular, mast cell						1 (25%)
Lymph node, mesenteric	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, mast cell						1 (10%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
Brain	(10)	(0)	(0)	(0)	(0)	(10)
Cerebellum, mineralization	1 (10%)					

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(0)	(10)
Hemorrhage						1 (10%)
Inflammation, chronic active	2 (20%)					5 (50%)
Pleura	(0)	(0)	(0)	(0)	(0)	(1)
Inflammation, granulomatous						1 (100%)
Special Senses System						
Harderian gland	(10)	(0)	(0)	(0)	(0)	(10)
Inflammation, chronic						1 (10%)
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Accumulation, hyaline droplet	1 (10%)	10 (100%)	10 (100%)	10 (100%)	10 (100)%	10 (100%)
Casts granular		9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Nephropathy	9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Natural deaths						6
Survivors						
Terminal kill	10	10	10	10	10	4
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Mesentery	(1)	(1)	(3)	(0)	(0)	(0)
Fat, necrosis	1 (100%)	1 (100%)	3 (100%)			
Tongue	(0)	(1)	(0)	(0)	(0)	(0)
Cyst		1 (100%)				
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(10)	(10)
Cardiomyopathy	3 (30%)				2 (20%)	1 (10%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Vagina	(0)	(1)	(0)	(0)	(0)	(0)
Cyst		1 (100%)				
Hematopoietic System						
Lymph node, bronchial	(1)	(0)	(0)	(0)	(0)	(0)
Hemorrhage	1 (100%)					
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(10)	(9)
Inflammation, chronic active	4 (40%)				2 (20%)	5 (56%)
Alveolar epithelium, hyperplasia					1 (10%)	
Special Senses System						
Eye	(10)	(0)	(0)	(0)	(9)	(10)
Cataract					1 (11%)	
Retina, atrophy					1 (11%)	
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Nephropathy	1 (10%)				1 (10%)	

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal kill	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Gallbladder	(9)	(0)	(0)	(0)	(0)	(7)
Infiltration cellular, polymorphonuclear						1 (14%)
Pancreas	(10)	(0)	(0)	(1)	(0)	(10)
Duct, cyst				1 (100%)		
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(10)	(0)	(0)	(0)	(0)	(10)
Hypertrophy	2 (20%)					
General Body System						
None						
Genital System						
None						
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Dilatation			1 (10%)	1 (10%)		
Nephropathy			1 (10%)	1 (10%)		1 (10%)
Urinary bladder	(10)	(10)	(10)	(10)	(10)	(10)
Transitional epithelium, hyperplasia				7 (70%)	10 (100%)	10 (100%)

TABLE A4
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal kill	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Stomach, glandular	(10)	(0)	(0)	(0)	(0)	(10)
Mineralization						1 (10%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
Ovary	(10)	(0)	(0)	(0)	(0)	(10)
Teratoma benign	1 (10%)					
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(0)	(10)
Alveolar epithelium, hyperplasia						1 (10%)
Special Senses System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female Mice in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Nephropathy	2 (20%)		1 (10%)			1 (10%)
Urinary bladder	(10)	(10)	(10)	(10)	(10)	(10)
Transitional epithelium, hyperplasia				6 (60%)	10 (100%)	10 (100%)

APPENDIX B
GENETIC TOXICOLOGY

TABLE B1
Mutagenicity of α-Pinene in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA100						
	0	49 ± 2	57 ± 7	38 ± 4	70 ± 2	63 ± 4
	5		48 ± 2		123 ± 3	127 ± 6
	10	46 ± 5	49 ± 4	45 ± 6		
	25		52 ± 2			
	50	44 ± 4	29 ± 3	41 ± 13	65 ± 5	57 ± 5
	75		14 ± 1			
	100	63 ± 5		54 ± 15	80 ± 1	85 ± 15
	200	57 ± 4				
	250			19 ± 6		
	400	10 ± 1				
	500			12 ± 4	63 ± 6	48 ± 4
	1,000				58 ± 11	39 ± 3
	5,000				33 ± 3	90 ± 4
Trial summary		Negative	Negative	Negative	Negative	Negative
Positive control[b]		428 ± 39	387 ± 19	574 ± 1	738 ± 31	1,161 ± 65

Strain	Dose (µg/plate)	With 10% rat S9	With 10% rat S9
TA100 (continued)			
	0	56 ± 2	48 ± 10
	50		50 ± 7
	100		40 ± 4
	500	49 ± 4	52 ± 6
	1,000		38 ± 2
	1,500	46 ± 2	
	2,500	50 ± 2	
	5,000	21 ± 1	27 ± 2[c]
	10,000	11 ± 1	
Trial summary		Negative	Negative
Positive control		665 ± 29	510 ± 27

TABLE B1
Mutagenicity of α-Pinene in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA98						
	0	22 ± 3	15 ± 3	21 ± 2	30 ± 4	28 ± 5
	5		10 ± 3	23 ± 3		
	10	25 ± 3	9 ± 1	19 ± 4		
	20		8 ± 3			
	25			18 ± 0		
	30		6 ± 1			
	40		4 ± 1[d]			
	50	8 ± 2[c]		18 ± 3		20 ± 3
	75			9 ± 2		
	100	15 ± 2[c]				31 ± 3
	250	4 ± 1[c]				
	500	Toxic			32 ± 1	25 ± 2
	1,000					28 ± 5
	1,500				24 ± 5	
	2,500				21 ± 1	
	5,000				13 ± 3	15 ± 1[c]
	10,000				9 ± 1	
Trial summary		Negative	Negative	Negative	Negative	Negative
Positive control		468 ± 17	506 ± 17	295 ± 14	418 ± 22	1,008 ± 32

Strain	Dose (µg/plate)	With 10% rat S9
TA98 (continued)		
	0	22 ± 2
	50	22 ± 1
	100	21 ± 3
	500	19 ± 2
	1,000	35 ± 3
	5,000	7 ± 1
Trial summary		Negative
Positive control		372 ± 38

TABLE B1
Mutagenicity of α-Pinene in Bacterial Tester Strains

Strain	Dose (μg/plate)	Without S9	Without S9	Without S9	With 10% rat S9	With 10% rat S9
***Escherichia coli* WP2 *uvrA*/pKM101**						
	0	181 ± 46	136 ± 2	161 ± 7	266 ± 54	204 ± 12
	100	90 ± 8	139 ± 4	141 ± 5	210 ± 2	224 ± 4
	500	89 ± 2	175 ± 31	135 ± 12	208 ± 3	193 ± 13
	1,000	84 ± 19	156 ± 9	137 ± 3	287 ± 46	207 ± 17
	5,000	94 ± 5	148 ± 3	132 ± 4	162 ± 10	184 ± 21
	10,000	78 ± 5	165 ± 10	126 ± 2	174 ± 24	188 ± 13
Trial summary		Negative	Negative	Negative	Negative	Negative
Positive control		1,183 ± 140	930 ± 19	1,529 ± 121	710 ± 32	1,171 ± 10

[a] Study was performed at SITEK Research Laboratories using a modification of the protocol presented by Zeiger *et al*. (1992) and the same lot of α-pinene (4KB705) used in the 3-month studies. Data are presented as revertants/plate (mean ± standard error) from three plates. 0 μg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E.coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

[c] Slight toxicity

[d] Precipitate on plate

TABLE B2
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Treatment with α-Pinene by Inhalation for 3 Months[a]

	Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
Air[d]		5	1.6 ± 0.33		2.50 ± 0.39
α-Pinene	25	5	1.8 ± 0.30	0.3657	2.34 ± 0.19
	50	5	1.9 ± 0.53	0.3059	2.20 ± 0.26
	100	5	2.1 ± 0.43	0.2053	2.88 ± 0.31
	200	5	1.9 ± 0.29	0.3059	2.74 ± 0.19
	400	5	1.4 ± 0.40	0.6426	3.10 ± 0.20
			P=0.742[e]		
Female					
Air		5	1.4 ± 0.19		2.40 ± 0.19
α-Pinene	25	5	2.1 ± 0.43	0.1182	2.16 ± 0.26
	50	5	1.8 ± 0.25	0.2396	2.16 ± 0.20
	100	5	1.7 ± 0.44	0.2949	2.74 ± 0.36
	200	5	1.7 ± 0.30	0.2949	2.06 ± 0.29
	400	5	1.1 ± 0.19	0.7259	2.16 ± 0.06
			P=0.899		

[a] Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the chamber control group, significant at P≤0.005
[d] Chamber control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025

APPENDIX C
CLINICAL PATHOLOGY RESULTS

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
Hematology						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	9	10	10	9
Week 14	10	10	10	10	10	10
Hematocrit (spun) (%)						
Day 4	45.6 ± 0.3	45.1 ± 0.6	46.5 ± 0.6	45.5 ± 0.4	44.8 ± 0.5	44.4 ± 0.4
Day 23	47.9 ± 0.5	48.0 ± 0.6	47.4 ± 0.4	47.8 ± 0.4	47.7 ± 0.3	48.6 ± 0.5
Week 14	49.5 ± 0.5	48.3 ± 0.4	49.0 ± 0.3	48.3 ± 0.7*	47.6 ± 0.3**	47.7 ± 0.4**
Packed cell volume (mL/dL)						
Day 4	44.6 ± 0.4	44.1 ± 0.6	45.0 ± 0.6	44.2 ± 0.4	43.1 ± 0.3*	43.3 ± 0.4*
Day 23	47.4 ± 0.4	47.1 ± 0.5	46.5 ± 0.5	47.2 ± 0.4	46.9 ± 0.4	48.1 ± 0.4
Week 14	49.9 ± 0.5	48.9 ± 0.4	49.5 ± 0.3	48.3 ± 0.3*	48.0 ± 0.3**	47.8 ± 0.6**
Hemoglobin (g/dL)						
Day 4	13.5 ± 0.1	13.4 ± 0.2	13.7 ± 0.1	13.6 ± 0.2	13.3 ± 0.1	13.2 ± 0.1
Day 23	14.9 ± 0.1	14.9 ± 0.1	14.6 ± 0.1	15.0 ± 0.1	14.8 ± 0.1	15.1 ± 0.1
Week 14	15.7 ± 0.1	15.5 ± 0.1	15.5 ± 0.1	15.3 ± 0.1*	15.0 ± 0.1**	15.1 ± 0.2**
Erythrocytes (10^6/μL)						
Day 4	7.15 ± 0.09	7.12 ± 0.11	7.27 ± 0.07	7.26 ± 0.08	7.09 ± 0.07	7.06 ± 0.07
Day 23	8.06 ± 0.06	7.95 ± 0.07	7.84 ± 0.09	8.04 ± 0.08	7.92 ± 0.07	8.10 ± 0.06
Week 14	9.35 ± 0.07	9.09 ± 0.05	9.25 ± 0.06	8.94 ± 0.05**	8.92 ± 0.08**	8.86 ± 0.10**
Reticulocytes (10^3/μL)						
Day 4	555.1 ± 33.2	528.5 ± 25.8	576.3 ± 22.7	550.7 ± 22.1	595.2 ± 27.5	594.3 ± 27.2
Day 23	246.2 ± 4.8	247.5 ± 9.6	246.2 ± 13.4	239.4 ± 4.7	248.5 ± 12.8	237.0 ± 15.5
Week 14	198.3 ± 15.9	165.1 ± 12.7	220.8 ± 8.6[b]	218.1 ± 9.0	230.8 ± 17.1	238.0 ± 14.0
Nucleated erythrocytes/100 leukocytes						
Day 4	2.40 ± 0.52	2.50 ± 0.65	1.70 ± 0.21	1.80 ± 0.44	2.00 ± 0.33	3.80 ± 0.71
Day 23	0.60 ± 0.31	0.40 ± 0.22	0.11 ± 0.11	0.50 ± 0.17	0.20 ± 0.13	0.11 ± 0.11
Week 14	0.50 ± 0.22	0.30 ± 0.15	0.60 ± 0.22	0.40 ± 0.22	0.30 ± 0.15	0.70 ± 0.21
Mean cell volume (fL)						
Day 4	62.3 ± 0.3	62.0 ± 0.4	61.8 ± 0.3	60.9 ± 0.5	60.9 ± 0.4*	61.3 ± 0.4
Day 23	58.8 ± 0.2	59.2 ± 0.4	59.4 ± 0.2	58.8 ± 0.4	59.3 ± 0.4	59.4 ± 0.3
Week 14	53.4 ± 0.2	53.9 ± 0.4	53.5 ± 0.3	54.0 ± 0.4	53.9 ± 0.3	53.9 ± 0.3
Mean cell hemoglobin (pg)						
Day 4	18.8 ± 0.1	18.8 ± 0.1	18.9 ± 0.0	18.8 ± 0.1	18.8 ± 0.1	18.7 ± 0.0
Day 23	18.5 ± 0.1	18.7 ± 0.1	18.7 ± 0.1	18.6 ± 0.1	18.6 ± 0.1	18.6 ± 0.1
Week 14	16.9 ± 0.1	17.0 ± 0.1	16.8 ± 0.1	17.1 ± 0.1	16.9 ± 0.1	17.1 ± 0.1
Mean cell hemoglobin concentration (g/dL)						
Day 4	30.2 ± 0.1	30.4 ± 0.2	30.5 ± 0.1	30.9 ± 0.2*	30.9 ± 0.1**	30.5 ± 0.1
Day 23	31.5 ± 0.2	31.7 ± 0.2	31.4 ± 0.2	31.7 ± 0.2	31.5 ± 0.1	31.4 ± 0.2
Week 14	31.6 ± 0.1	31.6 ± 0.1	31.4 ± 0.1	31.5 ± 0.1	31.3 ± 0.1	31.7 ± 0.2
Platelets (10^3/μL)						
Day 4	895.4 ± 12.5	909.1 ± 16.4	895.6 ± 18.2	865.7 ± 15.8	915.4 ± 20.6	895.2 ± 20.8
Day 23	795.4 ± 17.4	783.0 ± 23.4	793.0 ± 13.3	804.6 ± 12.7[b]	853.2 ± 17.9	825.5 ± 14.1[c]
Week 14	648.9 ± 5.3	703.3 ± 14.8**	696.2 ± 10.9*	663.8 ± 10.2	664.1 ± 14.7	666.3 ± 16.0
Leukocytes (10^3/μL)						
Day 4	9.08 ± 0.45	8.94 ± 0.32	9.75 ± 0.44	9.04 ± 0.72	7.57 ± 0.36*	6.95 ± 0.25**
Day 23	6.29 ± 0.23	7.10 ± 0.25	7.44 ± 0.29	8.12 ± 0.42**	7.52 ± 0.64	7.20 ± 0.31
Week 14	7.01 ± 0.36	6.99 ± 0.39	7.86 ± 0.25	7.34 ± 0.37	6.71 ± 0.40	6.69 ± 0.47
Segmented neutrophils (10^3/μL)						
Day 4	0.87 ± 0.05	0.93 ± 0.04	1.01 ± 0.08	1.00 ± 0.11	0.87 ± 0.05	0.90 ± 0.04
Day 23	0.90 ± 0.05	0.94 ± 0.06	0.97 ± 0.05[d]	0.97 ± 0.05	1.04 ± 0.05	0.95 ± 0.05[d]
Week 14	1.20 ± 0.04	1.26 ± 0.05	1.34 ± 0.05	1.22 ± 0.04	1.19 ± 0.04	1.20 ± 0.09

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
Hematology (continued)						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	9	10	10	9
Week 14	10	10	10	10	10	10
Bands (10^3/μL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)						
Day 4	7.99 ± 0.40	7.82 ± 0.30	8.39 ± 0.38	7.75 ± 0.61	6.44 ± 0.33**	5.88 ± 0.24**
Day 23	5.25 ± 0.23	5.91 ± 0.25	6.38 ± 0.22[d]	6.89 ± 0.40*	6.10 ± 0.57	6.06 ± 0.27
Week 14	5.36 ± 0.35	5.31 ± 0.39	6.18 ± 0.25	5.66 ± 0.40	5.12 ± 0.41	4.93 ± 0.38
Monocytes (10^3/μL)						
Day 4	0.12 ± 0.03	0.10 ± 0.03	0.26 ± 0.03*	0.17 ± 0.03	0.15 ± 0.03	0.09 ± 0.03
Day 23	0.07 ± 0.02	0.16 ± 0.04	0.07 ± 0.03[d]	0.18 ± 0.06	0.31 ± 0.12	0.22 ± 0.07[d]
Week 14	0.36 ± 0.08	0.32 ± 0.09	0.23 ± 0.11	0.35 ± 0.08	0.28 ± 0.08	0.44 ± 0.12
Basophils (10^3/μL)						
Day 4	0.008 ± 0.002	0.006 ± 0.002	0.010 ± 0.003	0.010 ± 0.001	0.007 ± 0.002	0.004 ± 0.002
Day 23	0.005 ± 0.002	0.004 ± 0.002	0.004 ± 0.002[d]	0.011 ± 0.005	0.008 ± 0.003	0.006 ± 0.003[d]
Week 14	0.010 ± 0.003	0.007 ± 0.003	0.006 ± 0.003	0.009 ± 0.003	0.008 ± 0.002	0.006 ± 0.002
Eosinophils (10^3/μL)						
Day 4	0.09 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.08 ± 0.01
Day 23	0.06 ± 0.01	0.09 ± 0.01	0.08 ± 0.02[d]	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01[d]
Week 14	0.09 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Clinical Chemistry						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 4	7.5 ± 0.4	7.8 ± 0.4	7.5 ± 0.3	7.4 ± 0.3	7.0 ± 0.4	7.5 ± 0.4
Day 23	9.9 ± 0.5	8.9 ± 0.4	9.1 ± 0.2	9.5 ± 0.3	9.8 ± 0.4	11.4 ± 0.6
Week 14	12.3 ± 0.3	13.7 ± 0.3*	12.8 ± 0.3	13.3 ± 0.2	13.3 ± 0.3	13.6 ± 0.4*
Creatinine (mg/dL)						
Day 4	0.29 ± 0.01	0.26 ± 0.02	0.23 ± 0.02*	0.25 ± 0.02	0.25 ± 0.02	0.24 ± 0.02
Day 23	0.30 ± 0.00	0.32 ± 0.01	0.32 ± 0.03	0.31 ± 0.01	0.36 ± 0.02**	0.38 ± 0.01**
Week 14	0.37 ± 0.02	0.37 ± 0.02	0.37 ± 0.03	0.39 ± 0.02	0.39 ± 0.01	0.40 ± 0.03
Glucose (mg/dL)						
Day 4	137 ± 3	134 ± 1	133 ± 5	137 ± 3	139 ± 6	130 ± 2
Day 23	145 ± 12	126 ± 7	134 ± 9	127 ± 5	117 ± 4	116 ± 5
Week 14	127 ± 2	130 ± 3	124 ± 2	129 ± 3	136 ± 6	128 ± 3
Total protein (g/dL)						
Day 4	6.0 ± 0.0	6.0 ± 0.1	6.1 ± 0.1	6.0 ± 0.0	6.1 ± 0.1	6.1 ± 0.0
Day 23	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.8 ± 0.1**	6.8 ± 0.1**
Week 14	7.4 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	7.5 ± 0.0
Albumin (g/dL)						
Day 4	4.3 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.3 ± 0.0	4.4 ± 0.0	4.4 ± 0.0
Day 23	4.6 ± 0.0	4.6 ± 0.0	4.5 ± 0.0	4.5 ± 0.1	4.7 ± 0.0	4.7 ± 0.1
Week 14	4.9 ± 0.1	4.9 ± 0.0	4.9 ± 0.1	4.8 ± 0.0	4.9 ± 0.0	4.9 ± 0.0

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
Clinical Chemistry (continued)						
n	10	10	10	10	10	10
Globulin (g/dL)						
Day 4	1.7 ± 0.0	1.7 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
Day 23	1.9 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.0**	2.1 ± 0.0**
Week 14	2.6 ± 0.0	2.5 ± 0.0	2.6 ± 0.0	2.5 ± 0.0	2.6 ± 0.0	2.6 ± 0.0
A/G ratio						
Day 4	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.4 ± 0.0
Day 23	2.4 ± 0.0	2.4 ± 0.0	2.3 ± 0.1	2.3 ± 0.0	2.3 ± 0.0	2.2 ± 0.0**
Week 14	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	2.0 ± 0.0
Alanine aminotransferase (IU/L)						
Day 4	57 ± 1	57 ± 1	55 ± 1	53 ± 1	55 ± 1	52 ± 1**
Day 23	41 ± 1	41 ± 1	41 ± 1	39 ± 2	38 ± 1	35 ± 0**
Week 14	85 ± 3	83 ± 3	70 ± 3**	60 ± 2**	56 ± 2**	51 ± 2**
Alkaline phosphatase (IU/L)						
Day 4	575 ± 7	578 ± 10	566 ± 10	566 ± 11	554 ± 7	546 ± 11*
Day 23	406 ± 6	423 ± 11	433 ± 9	407 ± 11	420 ± 8	404 ± 12
Week 14	223 ± 5	227 ± 7	211 ± 4	200 ± 3**	204 ± 4**	199 ± 6**
Creatine kinase (IU/L)						
Day 4	545 ± 121	507 ± 42	430 ± 52	449 ± 56	515 ± 54	434 ± 44
Day 23	404 ± 37	390 ± 40	409 ± 66	393 ± 37	354 ± 30	413 ± 45
Week 14	171 ± 8	186 ± 18	144 ± 14	155 ± 13	150 ± 14	183 ± 15
Sorbitol dehydrogenase (IU/L)						
Day 4	13 ± 1	14 ± 0	13 ± 0	12 ± 0*	14 ± 1	13 ± 0
Day 23	14 ± 1	14 ± 1	16 ± 1	15 ± 1	18 ± 1**	15 ± 1
Week 14	24 ± 1	24 ± 1	22 ± 1	22 ± 1	21 ± 1*	20 ± 1**
Bile acids (μmol/L)						
Day 4	4.7 ± 0.4	4.7 ± 0.5	5.6 ± 0.8	4.6 ± 0.4	7.2 ± 1.3	4.6 ± 0.7
Day 23	5.7 ± 0.9	3.3 ± 0.2**	4.9 ± 0.6*	3.6 ± 0.3**	3.8 ± 0.3**	3.6 ± 0.7**
Week 14	3.3 ± 0.1	3.5 ± 0.4	3.4 ± 0.3	3.2 ± 0.1	3.8 ± 0.6	3.0 ± 0.1
Female						
Hematology						
n						
Day 4	10	10	9	10	9	10
Day 23	10	10	10	9	10	9
Week 14	10	10	10	10	10	4
Hematocrit (spun) (%)						
Day 4	47.7 ± 0.4	47.0 ± 0.2	46.6 ± 0.3	47.5 ± 0.3	47.1 ± 0.6	46.2 ± 0.4
Day 23	48.7 ± 0.4	49.2 ± 0.5	49.1 ± 0.4	49.2 ± 0.3	48.9 ± 0.5	49.7 ± 0.6
Week 14	48.9 ± 0.4	47.2 ± 0.4*	47.8 ± 0.2	48.3 ± 0.4	48.7 ± 0.4	50.9 ± 0.8
Packed cell volume (mL/dL)						
Day 4	46.7 ± 0.5	46.1 ± 0.3	46.0 ± 0.3	46.8 ± 0.4	46.1 ± 0.5	45.3 ± 0.5
Day 23	48.5 ± 0.4	49.0 ± 0.4	49.3 ± 0.3	49.2 ± 0.3	48.8 ± 0.3	50.0 ± 0.6*
Week 14	49.1 ± 0.3	48.6 ± 0.3	48.7 ± 0.3	49.0 ± 0.5	49.7 ± 0.4	52.7 ± 0.4
Hemoglobin (g/dL)						
Day 4	14.3 ± 0.1	14.2 ± 0.1	14.2 ± 0.1	14.5 ± 0.1	14.3 ± 0.2	14.1 ± 0.1
Day 23	15.3 ± 0.1	15.5 ± 0.1	15.5 ± 0.1	15.5 ± 0.1	15.3 ± 0.1	15.7 ± 0.2
Week 14	15.7 ± 0.1	15.4 ± 0.1	15.5 ± 0.1	15.6 ± 0.1	15.8 ± 0.1	16.7 ± 0.2

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Female						
Hematology (continued)						
n						
Day 4	10	10	9	10	9	10
Day 23	10	10	10	9	10	9
Week 14	10	10	10	10	10	4
Erythrocytes (10^6/μL)						
Day 4	7.64 ± 0.07	7.56 ± 0.05	7.56 ± 0.05	7.74 ± 0.07	7.67 ± 0.10	7.55 ± 0.08
Day 23	8.13 ± 0.08	8.13 ± 0.08	8.20 ± 0.07	8.24 ± 0.05	8.13 ± 0.06	8.31 ± 0.09
Week 14	8.67 ± 0.06	8.53 ± 0.05	8.54 ± 0.06	8.62 ± 0.09	8.71 ± 0.06	9.23 ± 0.09
Reticulocytes (10^3/μL)						
Day 4	402.3 ± 27.2	360.7 ± 22.5	384.6 ± 24.4	393.6 ± 29.7	392.2 ± 24.4	361.1 ± 13.7
Day 23	228.6 ± 13.4	219.3 ± 14.2	211.5 ± 9.3	210.1 ± 13.4	212.2 ± 8.2	227.8 ± 14.6
Week 14	197.6 ± 11.2	183.5 ± 7.7	176.0 ± 15.9	160.4 ± 13.9	200.3 ± 6.5	163.5 ± 24.4
Nucleated erythrocytes/100 leukocytes						
Day 4	0.90 ± 0.31	0.40 ± 0.31	0.67 ± 0.24	0.70 ± 0.21	0.67 ± 0.24	1.00 ± 0.26
Day 23	0.50 ± 0.22	0.30 ± 0.15	0.70 ± 0.26	0.33 ± 0.24	0.40 ± 0.16	0.00 ± 0.00
Week 14	0.70 ± 0.30	0.30 ± 0.21	0.40 ± 0.16	0.20 ± 0.13	0.40 ± 0.22	0.00 ± 0.00
Mean cell volume (fL)						
Day 4	61.1 ± 0.3	60.9 ± 0.3	60.9 ± 0.2	60.5 ± 0.3	60.2 ± 0.5	60.0 ± 0.3*
Day 23	59.6 ± 0.3	60.3 ± 0.3	60.1 ± 0.3	59.7 ± 0.3	60.1 ± 0.3	60.2 ± 0.2
Week 14	56.6 ± 0.2	56.9 ± 0.1	57.0 ± 0.1	56.9 ± 0.1	57.0 ± 0.2	57.1 ± 0.3
Mean cell hemoglobin (pg)						
Day 4	18.7 ± 0.1	18.8 ± 0.1	18.8 ± 0.1	18.7 ± 0.1	18.7 ± 0.1	18.7 ± 0.1
Day 23	18.8 ± 0.1	19.0 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.8 ± 0.1	18.9 ± 0.1
Week 14	18.1 ± 0.0	18.1 ± 0.1	18.2 ± 0.1	18.1 ± 0.1	18.1 ± 0.0	18.1 ± 0.0
Mean cell hemoglobin concentration (g/dL)						
Day 4	30.6 ± 0.2	30.8 ± 0.1	30.8 ± 0.1	31.0 ± 0.2	31.1 ± 0.2	31.2 ± 0.2
Day 23	31.6 ± 0.2	31.5 ± 0.1	31.4 ± 0.1	31.6 ± 0.1	31.4 ± 0.1	31.4 ± 0.1
Week 14	32.1 ± 0.1	31.8 ± 0.1	31.9 ± 0.1	31.9 ± 0.1	31.8 ± 0.1	31.7 ± 0.2
Platelets (10^3/μL)						
Day 4	820.1 ± 18.0	831.2 ± 19.1	837.7 ± 22.6	797.5 ± 26.6	777.3 ± 23.1	845.4 ± 25.2
Day 23	764.7 ± 15.4	750.2 ± 11.8	738.4 ± 16.7	753.3 ± 10.1	778.8 ± 10.3	789.7 ± 15.9
Week 14	689.3 ± 7.2	660.9 ± 11.1*	665.4 ± 16.6*	664.1 ± 8.9*	649.0 ± 4.6**[b]	580.0 ± 31.5**
Leukocytes (10^3/μL)						
Day 4	10.52 ± 0.52	10.89 ± 0.26	10.25 ± 0.34	11.26 ± 0.61	10.39 ± 0.63	8.52 ± 0.68
Day 23	7.96 ± 0.36	8.01 ± 0.24	7.87 ± 0.43	8.04 ± 0.39	7.78 ± 0.56	6.84 ± 0.48
Week 14	5.86 ± 0.27	5.70 ± 0.24	6.05 ± 0.29	5.60 ± 0.29	5.22 ± 0.33	6.08 ± 0.58
Segmented neutrophils (10^3/μL)						
Day 4	0.89 ± 0.02	0.95 ± 0.04	0.99 ± 0.07	1.19 ± 0.07**	1.07 ± 0.11[d]	0.92 ± 0.08
Day 23	0.97 ± 0.05	0.88 ± 0.04	0.83 ± 0.04	0.88 ± 0.07	0.93 ± 0.08	0.80 ± 0.07*
Week 14	0.93 ± 0.08	0.90 ± 0.07	1.02 ± 0.10	0.83 ± 0.06	0.84 ± 0.07	0.80 ± 0.14
Bands (10^3/μL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)						
Day 4	9.34 ± 0.52	9.58 ± 0.25	8.90 ± 0.30	9.76 ± 0.59	9.19 ± 0.51[d]	7.34 ± 0.62
Day 23	6.79 ± 0.35	6.86 ± 0.20	6.83 ± 0.41	6.96 ± 0.38	6.62 ± 0.54	5.83 ± 0.42
Week 14	4.67 ± 0.24	4.44 ± 0.25	4.67 ± 0.23	4.36 ± 0.28	4.17 ± 0.29	4.93 ± 0.53
Monocytes (10^3/μL)						
Day 4	0.17 ± 0.04	0.23 ± 0.04	0.24 ± 0.06	0.17 ± 0.07	0.11 ± 0.04	0.11 ± 0.02
Day 23	0.10 ± 0.03	0.17 ± 0.02	0.11 ± 0.02	0.10 ± 0.04	0.13 ± 0.03	0.11 ± 0.02
Week 14	0.19 ± 0.06	0.28 ± 0.05	0.27 ± 0.05	0.33 ± 0.06	0.14 ± 0.04	0.27 ± 0.10

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Female						
Hematology (continued)						
n						
Day 4	10	10	9	10	9	10
Day 23	10	10	10	9	10	9
Week 14	10	10	10	10	10	4
Basophils (10^3/μL)						
Day 4	0.013 ± 0.002	0.014 ± 0.002	0.009 ± 0.003	0.013 ± 0.004	0.008 ± 0.001	0.015 ± 0.005
Day 23	0.009 ± 0.003	0.011 ± 0.002	0.011 ± 0.003	0.006 ± 0.002	0.005 ± 0.002	0.006 ± 0.002
Week 14	0.003 ± 0.002	0.005 ± 0.002	0.004 ± 0.002	0.005 ± 0.002	0.002 ± 0.001	0.005 ± 0.003
Eosinophils (10^3/μL)						
Day 4	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.15 ± 0.02	0.14 ± 0.02
Day 23	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.09 ± 0.01
Week 14	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Clinical Chemistry						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	10	10	10	9
Week 14	10	10	10	10	10	4
Urea nitrogen (mg/dL)						
Day 4	7.8 ± 0.4	8.5 ± 0.3	8.1 ± 0.3	8.3 ± 0.4	9.1 ± 0.3*	8.6 ± 0.3
Day 23	11.5 ± 0.3	11.8 ± 0.4	11.5 ± 0.4	10.6 ± 0.4	10.8 ± 0.3	9.1 ± 0.4**
Week 14	14.1 ± 0.4	14.4 ± 0.4	13.0 ± 0.5	13.6 ± 0.5	13.4 ± 0.5	11.3 ± 0.5*
Creatinine (mg/dL)						
Day 4	0.29 ± 0.01	0.28 ± 0.01	0.29 ± 0.02	0.26 ± 0.02	0.28 ± 0.01	0.26 ± 0.02
Day 23	0.31 ± 0.01	0.30 ± 0.00	0.28 ± 0.01	0.30 ± 0.00	0.30 ± 0.00	0.31 ± 0.01
Week 14	0.37 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	0.38 ± 0.01	0.34 ± 0.02	0.35 ± 0.03
Glucose (mg/dL)						
Day 4	138 ± 2	135 ± 2	136 ± 4	136 ± 2	139 ± 5	130 ± 2
Day 23	127 ± 3	123 ± 6	133 ± 5	123 ± 3	122 ± 3	122 ± 5
Week 14	141 ± 8	131 ± 5	123 ± 2	133 ± 3	131 ± 4	114 ± 12
Total protein (g/dL)						
Day 4	5.9 ± 0.0	6.0 ± 0.1	6.1 ± 0.0	6.0 ± 0.0	6.1 ± 0.1*	6.1 ± 0.0
Day 23	6.3 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.6 ± 0.1
Week 14	7.5 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.5 ± 0.1	7.2 ± 0.1
Albumin (g/dL)						
Day 4	4.3 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.4 ± 0.0
Day 23	4.5 ± 0.0	4.6 ± 0.0	4.6 ± 0.0	4.6 ± 0.1	4.6 ± 0.0	4.7 ± 0.1
Week 14	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.3 ± 0.0	5.2 ± 0.0	5.0 ± 0.1
Globulin (g/dL)						
Day 4	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.7 ± 0.0	1.7 ± 0.0*	1.6 ± 0.0
Day 23	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.9 ± 0.0*	1.9 ± 0.0*	2.0 ± 0.0**
Week 14	2.3 ± 0.1	2.2 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.4 ± 0.0	2.2 ± 0.1
A/G ratio						
Day 4	2.8 ± 0.0	2.7 ± 0.1	2.7 ± 0.0	2.7 ± 0.1	2.6 ± 0.1	2.7 ± 0.0
Day 23	2.6 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.4 ± 0.0	2.5 ± 0.0	2.4 ± 0.0**
Week 14	2.3 ± 0.1	2.4 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.2 ± 0.0	2.4 ± 0.1
Alanine aminotransferase (IU/L)						
Day 4	47 ± 1	49 ± 1	49 ± 1	46 ± 1	45 ± 1	44 ± 2
Day 23	35 ± 1	36 ± 1	35 ± 1	36 ± 1	34 ± 1	31 ± 1
Week 14	69 ± 4	65 ± 5	55 ± 3**	56 ± 4*	47 ± 2**	49 ± 5**

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Inhalation Study of α-Pinene

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Female						
Clinical Chemistry						
n						
Day 4	10	10	10	10	10	10
Day 23	10	10	10	10	10	9
Week 14	10	10	10	10	10	4
Alkaline phosphatase (IU/L)						
Day 4	487 ± 8	493 ± 10	475 ± 6	468 ± 7	454 ± 5**	457 ± 8**
Day 23	305 ± 5	311 ± 8	304 ± 5	302 ± 8	289 ± 8	289 ± 7
Week 14	197 ± 6	182 ± 4	182 ± 8	177 ± 8**	181 ± 5*	164 ± 13*
Creatine kinase (IU/L)						
Day 4	364 ± 20[b]	332 ± 27	388 ± 29[b]	443 ± 74	460 ± 39[b]	375 ± 48
Day 23	299 ± 30	305 ± 27	292 ± 41	369 ± 43	338 ± 26	250 ± 16
Week 14	162 ± 16	165 ± 38	172 ± 22	139 ± 14	170 ± 22	145 ± 26
Sorbitol dehydrogenase (IU/L)						
Day 4	13 ± 1	13 ± 0	14 ± 0	12 ± 0*	11 ± 1*	12 ± 0*
Day 23	14 ± 0	15 ± 1	15 ± 1	15 ± 1	14 ± 1	16 ± 0
Week 14	21 ± 1	20 ± 1	18 ± 1	17 ± 1	17 ± 1	18 ± 1
Bile acids (μmol/L)						
Day 4	5.3 ± 0.5	5.0 ± 0.5	6.5 ± 1.1	5.8 ± 0.6	6.8 ± 1.3	4.9 ± 0.5
Day 23	4.0 ± 0.3	4.7 ± 0.4	5.4 ± 0.7	4.5 ± 0.4	3.9 ± 0.4	4.0 ± 0.7
Week 14	9.1 ± 2.3	4.9 ± 0.5**	4.7 ± 0.4**	4.3 ± 0.3**	5.1 ± 1.1**	16.9 ± 4.7*

* Significantly different (P≤0.05) from the chamber control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=8
[d] n=10

TABLE C2
Hematology Data for Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
n	10	10	10	10	10	10
Male						
Hematocrit (spun) (%)	51.3 ± 0.3	50.5 ± 0.4	50.1 ± 0.3	51.1 ± 0.3	50.9 ± 0.4	49.8 ± 0.3*
Packed cell volume (mL/dL)	51.8 ± 0.2	51.6 ± 0.3	50.7 ± 0.4	52.1 ± 0.4	52.2 ± 0.4	51.1 ± 0.4
Hemoglobin (g/dL)	16.0 ± 0.1	16.0 ± 0.1	15.7 ± 0.1	16.0 ± 0.0	16.1 ± 0.1	15.7 ± 0.1
Erythrocytes (10^6/μL)	10.51 ± 0.06	10.47 ± 0.06	10.23 ± 0.09	10.52 ± 0.04	10.55 ± 0.08	10.10 ± 0.07**
Reticulocytes (10^3/μL)	223.7 ± 19.4	200.3 ± 14.9	193.9 ± 16.4	205.2 ± 13.0	214.3 ± 16.4	202.2 ± 15.9
Nucleated erythrocytes /100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	49.3 ± 0.3	49.2 ± 0.2	49.6 ± 0.2	49.4 ± 0.2	49.6 ± 0.2	50.6 ± 0.2**
Mean cell hemoglobin (pg)	15.3 ± 0.1	15.2 ± 0.1	15.4 ± 0.1	15.2 ± 0.0	15.2 ± 0.0	15.6 ± 0.1*
Mean cell hemoglobin concentration (g/dL)	31.0 ± 0.2	31.0 ± 0.1	31.0 ± 0.1	30.8 ± 0.2	30.7 ± 0.1	30.8 ± 0.1
Platelets (10^3/μL)	859.7 ± 23.1	863.1 ± 15.5	853.1 ± 18.3	864.3 ± 28.1	872.4 ± 22.9	895.9 ± 19.9
Leukocytes (10^3/μL)	3.10 ± 0.40	2.94 ± 0.43	2.03 ± 0.26	2.47 ± 0.15	2.31 ± 0.26	1.87 ± 0.17*
Segmented neutrophils (10^3/μL)	0.42 ± 0.07	0.47 ± 0.06	0.21 ± 0.02*	0.37 ± 0.05	0.32 ± 0.04	0.29 ± 0.02
Bands (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)	2.60 ± 0.34	2.41 ± 0.39	1.73 ± 0.23	2.03 ± 0.13	1.93 ± 0.22	1.48 ± 0.14*
Monocytes (10^3/μL)	0.03 ± 0.01	0.02 ± 0.00	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.02	0.08 ± 0.02
Basophils (10^3/μL)	0.020 ± 0.006	0.010 ± 0.003	0.014 ± 0.003	0.010 ± 0.003	0.008 ± 0.001	0.011 ± 0.003
Eosinophils (10^3/μL)	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Howell-Jolly bodies (% erythrocytes)	0.10 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Female						
Hematocrit (spun) (%)	49.6 ± 0.3	50.1 ± 0.4	49.4 ± 0.5	50.1 ± 0.3	48.9 ± 0.3	48.3 ± 0.3*
Packed cell volume (mL/dL)	50.3 ± 0.2	50.9 ± 0.4	50.0 ± 0.6	50.9 ± 0.3	49.5 ± 0.3	49.0 ± 0.3*
Hemoglobin (g/dL)	15.8 ± 0.1	16.0 ± 0.1	15.7 ± 0.2	15.9 ± 0.1	15.5 ± 0.1	15.5 ± 0.1*
Erythrocytes (10^6/μL)	10.21 ± 0.05	10.26 ± 0.06	10.10 ± 0.11	10.14 ± 0.06	9.96 ± 0.09*	9.85 ± 0.08**
Reticulocytes (10^3/μL)	269.5 ± 15.4	248.9 ± 14.9	251.9 ± 16.5	282.5 ± 18.3	240.1 ± 20.8	251.2 ± 15.3
Nucleated erythrocytes /100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	49.3 ± 0.2	49.7 ± 0.2	49.5 ± 0.3	50.1 ± 0.2*	49.7 ± 0.2	49.7 ± 0.2
Mean cell hemoglobin (pg)	15.5 ± 0.1	15.6 ± 0.1	15.5 ± 0.1	15.6 ± 0.1	15.6 ± 0.1	15.7 ± 0.1
Mean cell hemoglobin concentration (g/dL)	31.5 ± 0.2	31.4 ± 0.1	31.4 ± 0.2	31.1 ± 0.1	31.4 ± 0.1	31.6 ± 0.1
Platelets (10^3/μL)	772.3 ± 13.7	773.5 ± 22.8	771.2 ± 17.4	738.3 ± 29.9	800.4 ± 18.1	827.7 ± 15.2
Leukocytes (10^3/μL)	3.65 ± 0.35	3.10 ± 0.27	3.34 ± 0.32	2.80 ± 0.29	3.11 ± 0.32	3.16 ± 0.34
Segmented neutrophils (10^3/μL)	0.46 ± 0.05	0.35 ± 0.05	0.45 ± 0.04	0.31 ± 0.05	0.41 ± 0.06	0.38 ± 0.06
Bands (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)	3.09 ± 0.30	2.65 ± 0.23	2.78 ± 0.30	2.39 ± 0.25	2.60 ± 0.26	2.66 ± 0.27
Monocytes (10^3/μL)	0.04 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.07 ± 0.02
Basophils (10^3/μL)	0.019 ± 0.007	0.015 ± 0.003	0.019 ± 0.003	0.019 ± 0.005	0.017 ± 0.005	0.014 ± 0.003
Eosinophils (10^3/μL)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Howell-Jolly bodies (% erythrocytes)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0

* Significantly different ($P \leq 0.05$) from the chamber control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX D
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 2-Week Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm	800 ppm	1,600 ppm
n	5	5	5	5	0[b]	0[b]
Male						
Necropsy body wt	172 ± 3	171 ± 2	173 ± 7	176 ± 4		
Heart						
Absolute	0.620 ± 0.008	0.614 ± 0.006	0.592 ± 0.028	0.620 ± 0.018		
Relative	3.602 ± 0.098	3.584 ± 0.033	3.418 ± 0.039	3.534 ± 0.059		
R. Kidney						
Absolute	0.714 ± 0.014	0.758 ± 0.020	0.790 ± 0.044	0.796 ± 0.026		
Relative	4.142 ± 0.075	4.425 ± 0.117*	4.556 ± 0.098**	4.535 ± 0.069**		
Liver						
Absolute	7.988 ± 0.154	8.064 ± 0.196	8.284 ± 0.410	9.668 ± 0.422**		
Relative	46.331 ± 0.589	47.091 ± 1.287	47.824 ± 0.791	55.069 ± 1.780**		
Lung						
Absolute	1.294 ± 0.119	1.216 ± 0.067	1.228 ± 0.060	1.566 ± 0.075		
Relative	7.504 ± 0.660	7.081 ± 0.309	7.100 ± 0.245	8.971 ± 0.568		
R. Testis						
Absolute	0.994 ± 0.015	0.984 ± 0.016	0.981 ± 0.034	0.991 ± 0.016		
Relative	5.770 ± 0.130	5.744 ± 0.064	5.681 ± 0.157	5.654 ± 0.085		
Thymus						
Absolute	0.403 ± 0.012	0.439 ± 0.016	0.427 ± 0.016	0.426 ± 0.007		
Relative	2.344 ± 0.095	2.565 ± 0.106	2.477 ± 0.096	2.431 ± 0.037		
Female						
Necropsy body wt	125 ± 3	130 ± 3	129 ± 2	118 ± 2		
Heart						
Absolute	0.466 ± 0.012	0.484 ± 0.022	0.480 ± 0.008	0.444 ± 0.017		
Relative	3.728 ± 0.089	3.727 ± 0.080	3.736 ± 0.073	3.752 ± 0.091		
R. Kidney						
Absolute	0.530 ± 0.021[c]	0.586 ± 0.016	0.602 ± 0.007*	0.578 ± 0.017		
Relative	4.220 ± 0.058[c]	4.521 ± 0.038	4.686 ± 0.071**	4.896 ± 0.166**		
Liver						
Absolute	4.854 ± 0.194	5.404 ± 0.260	5.764 ± 0.051**	5.244 ± 0.138		
Relative	38.750 ± 0.728	41.602 ± 1.031*	44.888 ± 0.926**	44.379 ± 1.012**		
Lung						
Absolute	0.850 ± 0.022	1.066 ± 0.041**	1.042 ± 0.046*	0.862 ± 0.060		
Relative	6.808 ± 0.233	8.221 ± 0.223*	8.114 ± 0.371	7.315 ± 0.572		
Thymus						
Absolute	0.317 ± 0.003	0.335 ± 0.006	0.361 ± 0.011*	0.285 ± 0.017		
Relative	2.535 ± 0.052	2.590 ± 0.059	2.807 ± 0.062	2.413 ± 0.149		

* Significantly different (P≤0.05) from the chamber control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] No data were available for the 800 and 1,600 ppm groups due to 100% mortality.
[c] n=4

TABLE D2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
Male						
n	10	10	10	10	10	10
Necropsy body wt	335 ± 6	329 ± 11	333 ± 6	334 ± 7	330 ± 4	322 ± 6
Heart						
Absolute	0.918 ± 0.018	0.852 ± 0.029	0.875 ± 0.020	0.888 ± 0.022	0.855 ± 0.009	0.893 ± 0.019
Relative	2.739 ± 0.036	2.589 ± 0.031*	2.630 ± 0.045	2.659 ± 0.034	2.591 ± 0.032*	2.777 ± 0.034
R. Kidney						
Absolute	1.025 ± 0.019	1.012 ± 0.037	1.061 ± 0.026	1.137 ± 0.027**	1.209 ± 0.020**	1.286 ± 0.039**
Relative	3.058 ± 0.038	3.073 ± 0.037	3.186 ± 0.042*	3.405 ± 0.036**	3.660 ± 0.040**	3.991 ± 0.056**
Liver						
Absolute	10.54 ± 0.27	10.31 ± 0.40	10.44 ± 0.32	11.08 ± 0.36	11.37 ± 0.26	11.87 ± 0.45*
Relative	31.402 ± 0.375	31.270 ± 0.317	31.298 ± 0.490	33.152 ± 0.569*	34.393 ± 0.531**	36.807 ± 0.864**
Lung						
Absolute	1.690 ± 0.117	1.518 ± 0.063	1.694 ± 0.067	1.751 ± 0.067	1.678 ± 0.046	1.809 ± 0.081
Relative	5.020 ± 0.279	4.632 ± 0.193	5.088 ± 0.172	5.260 ± 0.223	5.082 ± 0.129	5.629 ± 0.227
Spleen						
Absolute	0.628 ± 0.012	0.630 ± 0.013	0.663 ± 0.014	0.659 ± 0.009	0.655 ± 0.010	0.677 ± 0.023*
Relative	1.874 ± 0.028	1.925 ± 0.045	1.997 ± 0.058	1.978 ± 0.030	1.983 ± 0.022	2.103 ± 0.057**
R. Testis						
Absolute	1.380 ± 0.019	1.303 ± 0.022	1.375 ± 0.019	1.347 ± 0.022	1.326 ± 0.028	1.378 ± 0.023
Relative	4.121 ± 0.065	3.983 ± 0.090	4.138 ± 0.074	4.040 ± 0.050	4.013 ± 0.074	4.291 ± 0.079
Thymus						
Absolute	0.416 ± 0.016	0.384 ± 0.019	0.400 ± 0.018	0.412 ± 0.011	0.409 ± 0.016	0.369 ± 0.018
Relative	1.241 ± 0.049	1.175 ± 0.068	1.208 ± 0.059	1.237 ± 0.039	1.237 ± 0.041	1.150 ± 0.054
Female						
n	10	10	10	10	10	4
Necropsy body wt	194 ± 3	199 ± 4	206 ± 4	199 ± 3	201 ± 3	159 ± 5**
Heart						
Absolute	0.584 ± 0.010	0.612 ± 0.012	0.618 ± 0.010	0.629 ± 0.012*	0.638 ± 0.011**	0.530 ± 0.006*
Relative	3.010 ± 0.039	3.081 ± 0.054	3.002 ± 0.041	3.156 ± 0.034*	3.175 ± 0.049*	3.349 ± 0.084**
R. Kidney						
Absolute	0.618 ± 0.011	0.641 ± 0.009	0.680 ± 0.013**	0.659 ± 0.015	0.679 ± 0.014**	0.595 ± 0.021
Relative	3.185 ± 0.040	3.230 ± 0.062	3.301 ± 0.041	3.307 ± 0.058	3.376 ± 0.050*	3.757 ± 0.138**
Liver						
Absolute	5.486 ± 0.179	5.990 ± 0.121	6.270 ± 0.115**	6.269 ± 0.151**	6.424 ± 0.144**	4.840 ± 0.247
Relative	28.216 ± 0.637	30.152 ± 0.550**	30.438 ± 0.319**	31.459 ± 0.586**	31.916 ± 0.317**	30.470 ± 0.715**
Lung						
Absolute	1.064 ± 0.014	1.055 ± 0.026	1.139 ± 0.033	1.205 ± 0.066	1.171 ± 0.056	1.148 ± 0.070
Relative	5.488 ± 0.082	5.304 ± 0.088	5.524 ± 0.111	6.056 ± 0.343	5.809 ± 0.216	7.234 ± 0.384**
Spleen						
Absolute	0.391 ± 0.005	0.402 ± 0.005	0.411 ± 0.006	0.393 ± 0.006	0.402 ± 0.006	0.320 ± 0.009**
Relative	2.017 ± 0.033	2.026 ± 0.034	1.997 ± 0.032	1.975 ± 0.037	2.001 ± 0.025	2.020 ± 0.047
Thymus						
Absolute	0.347 ± 0.012	0.349 ± 0.010	0.352 ± 0.010	0.346 ± 0.010	0.330 ± 0.014	0.204 ± 0.010**
Relative	1.785 ± 0.054	1.751 ± 0.029	1.707 ± 0.041	1.739 ± 0.048	1.638 ± 0.058*	1.286 ± 0.035**

* Significantly different (P≤0.05) from the chamber control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 2-Week Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm	800 ppm	1,600 ppm
n	5	5	5	5	0[b]	0[b]
Male						
Necropsy body wt	27.9 ± 0.7	26.7 ± 0.7	26.6 ± 0.9	27.0 ± 0.6		
Heart						
Absolute	0.136 ± 0.006	0.124 ± 0.004	0.126 ± 0.005	0.136 ± 0.008		
Relative	4.872 ± 0.140	4.640 ± 0.127	4.743 ± 0.182	5.028 ± 0.223		
R. Kidney						
Absolute	0.234 ± 0.011	0.246 ± 0.009	0.236 ± 0.015	0.254 ± 0.012		
Relative	8.377 ± 0.226	9.194 ± 0.191	8.832 ± 0.297	9.401 ± 0.337*		
Liver						
Absolute	1.436 ± 0.066	1.394 ± 0.044	1.476 ± 0.069	1.672 ± 0.056*		
Relative	51.415 ± 1.352	52.117 ± 0.648	55.355 ± 0.721*	61.935 ± 1.281**		
Lung						
Absolute	0.184 ± 0.011	0.186 ± 0.007	0.200 ± 0.017	0.184 ± 0.006		
Relative	6.579 ± 0.260	6.953 ± 0.154	7.498 ± 0.510	6.814 ± 0.120		
R. Testis						
Absolute	0.099 ± 0.002	0.095 ± 0.004	0.089 ± 0.010	0.094 ± 0.002		
Relative	3.539 ± 0.048	3.536 ± 0.109	3.301 ± 0.287	3.478 ± 0.074		
Thymus						
Absolute	0.057 ± 0.007	0.048 ± 0.004	0.050 ± 0.003	0.049 ± 0.007		
Relative	2.030 ± 0.192	1.801 ± 0.131	1.882 ± 0.105	1.831 ± 0.256		
Female						
Necropsy body wt	23.0 ± 0.4	23.6 ± 0.5	23.2 ± 0.7	22.6 ± 0.5		
Heart						
Absolute	0.118 ± 0.004	0.122 ± 0.004	0.120 ± 0.003	0.118 ± 0.002		
Relative	5.135 ± 0.090	5.163 ± 0.137	5.165 ± 0.034	5.221 ± 0.137		
R. Kidney						
Absolute	0.166 ± 0.005	0.196 ± 0.007*	0.184 ± 0.008	0.180 ± 0.006		
Relative	7.225 ± 0.137	8.283 ± 0.132**	7.919 ± 0.273	7.952 ± 0.234		
Liver						
Absolute	1.230 ± 0.029	1.300 ± 0.033	1.320 ± 0.065	1.426 ± 0.036*		
Relative	53.557 ± 0.672	54.996 ± 0.772	56.718 ± 1.676	63.001 ± 0.995**		
Lung						
Absolute	0.182 ± 0.006	0.190 ± 0.005	0.188 ± 0.004	0.198 ± 0.015		
Relative	7.932 ± 0.261	8.033 ± 0.085	8.114 ± 0.273	8.756 ± 0.661		
Thymus						
Absolute	0.074 ± 0.003	0.073 ± 0.005	0.068 ± 0.003	0.060 ± 0.004		
Relative	3.220 ± 0.122	3.059 ± 0.157	2.913 ± 0.123	2.654 ± 0.114*		

* Significantly different (P≤0.05) from the chamber control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] No data were available for the 800 and 1,600 ppm groups due to 100% mortality.

TABLE D4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	25 ppm	50 ppm	100 ppm	200 ppm	400 ppm
n	10	10	10	10	10	10
Male						
Necropsy body wt	37.1 ± 0.6	36.9 ± 0.7	38.3 ± 0.9	35.9 ± 0.7	35.5 ± 1.0	36.2 ± 0.5
Heart						
Absolute	0.162 ± 0.003	0.157 ± 0.003	0.170 ± 0.005	0.159 ± 0.003	0.150 ± 0.003	0.159 ± 0.006
Relative	4.377 ± 0.112	4.267 ± 0.091	4.446 ± 0.138	4.441 ± 0.129	4.251 ± 0.095	4.386 ± 0.151
R. Kidney						
Absolute	0.330 ± 0.006	0.318 ± 0.009	0.336 ± 0.010	0.309 ± 0.008	0.295 ± 0.006*	0.307 ± 0.007*
Relative	8.903 ± 0.167	8.629 ± 0.208	8.793 ± 0.267	8.617 ± 0.205	8.348 ± 0.145	8.469 ± 0.155
Liver						
Absolute	1.617 ± 0.022	1.589 ± 0.028	1.702 ± 0.040	1.637 ± 0.024	1.660 ± 0.043	1.957 ± 0.057**
Relative	43.671 ± 0.880	43.123 ± 0.458	44.487 ± 0.806	45.651 ± 0.678	46.903 ± 0.750*	54.009 ± 1.465**
Lung						
Absolute	0.217 ± 0.004	0.212 ± 0.004	0.209 ± 0.007	0.234 ± 0.012	0.199 ± 0.004	0.211 ± 0.007
Relative	5.856 ± 0.117	5.767 ± 0.160	5.484 ± 0.226	6.525 ± 0.342	5.645 ± 0.176	5.816 ± 0.141
Spleen						
Absolute	0.072 ± 0.002	0.068 ± 0.002	0.076 ± 0.003	0.070 ± 0.001	0.069 ± 0.002	0.068 ± 0.002
Relative	1.944 ± 0.058	1.849 ± 0.059	1.991 ± 0.084	1.952 ± 0.041	1.953 ± 0.050	1.873 ± 0.053
R. Testis						
Absolute	0.117 ± 0.002	0.117 ± 0.002	0.116 ± 0.004	0.114 ± 0.002	0.112 ± 0.002	0.109 ± 0.002*
Relative	3.167 ± 0.062	3.170 ± 0.079	3.033 ± 0.064	3.178 ± 0.067	3.173 ± 0.069	3.017 ± 0.058
Thymus						
Absolute	0.066 ± 0.004	0.063 ± 0.004	0.067 ± 0.003	0.057 ± 0.001	0.062 ± 0.004	0.051 ± 0.003**
Relative	1.777 ± 0.081	1.699 ± 0.090	1.742 ± 0.063	1.591 ± 0.052	1.739 ± 0.115	1.397 ± 0.081**
Female						
Necropsy body wt	31.5 ± 0.6	30.3 ± 0.6	32.7 ± 0.7	31.5 ± 1.1	30.7 ± 0.6	30.6 ± 0.5
Heart						
Absolute	0.147 ± 0.002	0.146 ± 0.004	0.148 ± 0.006	0.157 ± 0.005	0.149 ± 0.004	0.147 ± 0.004
Relative	4.683 ± 0.121	4.816 ± 0.110	4.528 ± 0.139	5.027 ± 0.170	4.865 ± 0.134	4.802 ± 0.135
R. Kidney						
Absolute	0.208 ± 0.004	0.207 ± 0.003	0.206 ± 0.004	0.217 ± 0.007	0.212 ± 0.004	0.210 ± 0.003
Relative	6.620 ± 0.159	6.836 ± 0.122	6.321 ± 0.102	6.915 ± 0.116	6.913 ± 0.092	6.870 ± 0.132
Liver						
Absolute	1.466 ± 0.041	1.475 ± 0.053	1.442 ± 0.036	1.548 ± 0.053	1.587 ± 0.037	1.730 ± 0.032**
Relative	46.542 ± 0.988	48.567 ± 1.239	44.214 ± 0.880	49.280 ± 0.672*	51.728 ± 0.795**	56.511 ± 0.705**
Lung						
Absolute	0.253 ± 0.014	0.248 ± 0.010	0.235 ± 0.015	0.284 ± 0.015	0.249 ± 0.013	0.249 ± 0.013
Relative	8.057 ± 0.473	8.185 ± 0.337	7.172 ± 0.374	9.154 ± 0.600	8.159 ± 0.494	8.139 ± 0.413
Spleen						
Absolute	0.090 ± 0.002	0.090 ± 0.003	0.092 ± 0.002	0.097 ± 0.003	0.094 ± 0.003	0.094 ± 0.003
Relative	2.862 ± 0.071	2.968 ± 0.084	2.822 ± 0.067	3.097 ± 0.062	3.075 ± 0.126	3.072 ± 0.099
Thymus						
Absolute	0.053 ± 0.002	0.059 ± 0.002	0.058 ± 0.002	0.060 ± 0.003	0.055 ± 0.002	0.054 ± 0.002
Relative	1.692 ± 0.079	1.958 ± 0.076	1.790 ± 0.069	1.910 ± 0.071	1.794 ± 0.072	1.778 ± 0.076

* Significantly different (P≤0.05) from the chamber control group by Williams' test
** Significantly different (P≤0.01) from the chamber control group by Williams' or Dunnett's test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX E
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE E1
Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
n	10	10	9	10
Weights (g)				
Necropsy body wt	335 ± 6	334 ± 7	332 ± 4	322 ± 6
L. Cauda epididymis	0.1973 ± 0.0063	0.1923 ± 0.0062	0.1861 ± 0.0062	0.1802 ± 0.0057
L. Epididymis	0.4860 ± 0.0067	0.4724 ± 0.0094	0.4780 ± 0.0090	0.4650 ± 0.0092
L. Testis	1.4283 ± 0.0257	1.4061 ± 0.0160	1.4001 ± 0.0191	1.4337 ± 0.0213
Spermatid measurements				
Spermatid heads (10^3/mg testis)	129.3 ± 4.2	132.8 ± 3.7	136.7 ± 3.1	137.5 ± 3.3
Spermatid heads (10^6/testis)	167.5 ± 5.6	163.8 ± 5.1	168.3 ± 4.3	172.4 ± 3.5
Epididymal spermatozoal measurements				
Sperm motility (%)	91.73 ± 1.26	91.40 ± 0.93	91.24 ± 0.80	90.93 ± 0.89
Sperm (10^3/mg cauda epididymis)	615.0 ± 34.3	596.5 ± 31.8	526.3 ± 19.0	547.4 ± 14.0
Sperm (10^6/cauda epididymis)	120.89 ± 6.79	113.16 ± 3.11	97.52 ± 3.51**	98.40 ± 3.02**

** Significantly different (P≤0.01) from the chamber control group by Shirley's test.
[a] Data are presented as mean ± standard error.

TABLE E2
Estrous Cycle Characterization for Female Rats in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
Number weighed at necropsy	10	10	10	4
Necropsy body wt (g)	194 ± 3	199 ± 3	201 ± 3	159 ± 5**
Proportion of regular cycling females[b]	10/10	10/10	10/10	5/5
Estrous cycle length (days)	5.05 ± 0.05	5.00 ± 0.00	4.90 ± 0.10	6.00 ± 0.32**[c]
Estrous stages[d] (% of cycle)				
Diestrus	58.3	57.5	55.0	58.3
Proestrus	17.5	20.0	15.8	10.0
Estrus	20.8	20.8	20.8	18.3
Metestrus	3.3	1.7	8.3	13.3

** Significantly different (P≤0.01) from the chamber control group by Dunnett's test (body weights) or Dunn's test (estrous cycle length).
[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[b] Number of females with a regular cycle/number of females cycling
[c] n=5
[d] By multivariate analysis of variance, exposed females do not differ significantly from the chamber control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the chamber control group and each exposed group indicated exposed females did not spend significantly more time in the estrous stages than did the chamber control females.

Concentration (ppm)																		
0					P	E	D	D	D	P	E	D	D	D	P	E		
0			D	D	P	E	M	D	D	P	E	D	D	D				
0						E	M	D	D	P	E	D	D	D	P	E	D	
0		D	D	D	P	E	D	D	D	P	E	D	D					
0			D	D	P	E	D	D	D	P	E	D	D	D				
0		D	D	D	P	E	D	D	D	P	E	D	D					
0				P	E	E	D	D	D	P	E	D	D	D	P			
0						E	M	D	D	P	E	D	D	D	P	E	D	
0			D	D	E	E	D	D	D	P	E	D	D	D				
0			D	D	P	E	D	D	D	P	E	M	D	D				
100					P	E	D	D	D	P	E	D	D	D	P	E		
100		D	D	D	P	E	D	D	D	P	E	D	D					
100				D	P	E	D	D	D	P	E	D	D	D	P			
100						E	D	D	D	P	E	D	D	D	P	E	M	
100				D	P	E	D	D	D	P	E	D	D	D	P			
100						E	M	D	D	P	E	D	D	D	P	E	D	
100			D	D	P	E	D	D	D	P	E	D	D	D				
100		D	D	D	P	E	D	D	D	P	E	D	D					
100					P	E	D	D	D	P	E	D	D	D	P	E		
100						E	D	D	D	P	E	D	D	D	P	E	D	
200			D	D	P	E	D	D	D	P	E	D	D	D				
200				M	D	D	E	M	D	D	E	M	D	D	E			
200			D	D	E	E	M	D	D	P	E	M	D	D				
200					P	E	D	D	D	P	E	M	D	D	P	E		
200						E	D	D	D	P	E	D	D	D	P	E	D	
200				D	P	E	D	D	D	P	E	D	D	D	P			
200			D	D	E	E	D	D	D	P	E	D	D	D				
200				D	P	E	M	D	D	P	E	M	D	D	P			
200		D	D	D	P	E	D	D	D	P	E	M	D					
200		D	D	D	P	E	D	D	D	P	E	M	D					
400		M	D	D	P	E	D	D	D	P	E	D	D					
400				P	E	M	D	D	D	D	E	M	D	D	D			
400				D	E	M	D	D	D	D	E	M	D	D	D			
400			P	E	M	D	D	D	D	P	E	M	D	D				
400				D	P	E	D	D	D	D	E	E	M	D	D			

FIGURE E1
Individual Vaginal Cytology Plots for Female Rats in the 3-Month Inhalation Study of α-Pinene
D = diestrus, P = proestrus, E = estrus, M = metestrus. Cytology is aligned based on the second estrus observation.

TABLE E3
Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered α-Pinene by Inhalation for 3 Months

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	0.859	
Overall Tests	100 ppm vs. Chamber Controls	0.917	
Overall Tests	200 ppm vs. Chamber Controls	0.682	N
Overall Tests	400 ppm vs. Chamber Controls	0.44	N
Extended Estrus	Overall	0.913	
Extended Estrus	100 ppm vs. Chamber Controls	0.354	N
Extended Estrus	200 ppm vs. Chamber Controls	1	
Extended Estrus	400 ppm vs. Chamber Controls	1	
Extended Diestrus	Overall	0.704	
Extended Diestrus	100 ppm vs. Chamber Controls	0.561	
Extended Diestrus	200 ppm vs. Chamber Controls	0.666	N
Extended Diestrus	400 ppm vs. Chamber Controls	0.4	N
Extended Metestrus	Overall	1	
Extended Metestrus	100 ppm vs. Chamber Controls	1	
Extended Metestrus	200 ppm vs. Chamber Controls	1	
Extended Metestrus	400 ppm vs. Chamber Controls	1	
Extended Proestrus	Overall	1	
Extended Proestrus	100 ppm vs. Chamber Controls	1	
Extended Proestrus	200 ppm vs. Chamber Controls	1	
Extended Proestrus	400 ppm vs. Chamber Controls	1	
Skipped Estrus	Overall	1	
Skipped Estrus	100 ppm vs. Chamber Controls	1	
Skipped Estrus	200 ppm vs. Chamber Controls	1	
Skipped Estrus	400 ppm vs. Chamber Controls	1	
Skipped Diestrus	Overall	1	
Skipped Diestrus	100 ppm vs. Chamber Controls	1	
Skipped Diestrus	200 ppm vs. Chamber Controls	1	
Skipped Diestrus	400 ppm vs. Chamber Controls	1	

[a] N means that the treated group had a lower probability of transitioning to the relevant abnormal state (extended estrus, extended diestrus, extended metestrus, extended proestrus, skipped estrus, or skipped diestrus) than did the chamber control group.

TABLE E4
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	37.1 ± 0.6	35.9 ± 0.7	35.5 ± 1.0	36.2 ± 0.5
L. Cauda epididymis	0.0217 ± 0.0013	0.0173 ± 0.0007**	0.0187 ± 0.0010	0.0198 ± 0.0008
L. Epididymis	0.0527 ± 0.0013	0.0503 ± 0.0013	0.0485 ± 0.0019	0.0489 ± 0.0021
L. Testis	0.1144 ± 0.0021	0.1102 ± 0.0026	0.1068 ± 0.0019*	0.1073 ± 0.0018
Spermatid measurements				
Spermatid heads (10^3/mg testis)	190.9 ± 9.4[b]	197.8 ± 5.9[b]	214.5 ± 8.1*	202.7 ± 6.4
Spermatid heads (10^6/testis)	19.88 ± 1.09[b]	20.02 ± 0.53[b]	20.75 ± 0.65	19.48 ± 0.58
Epididymal spermatozoal measurements				
Sperm motility (%)	90.25 ± 0.34	88.31 ± 0.86	89.74 ± 0.80	87.95 ± 1.08
Sperm (10^3/mg cauda epididymis)	704.8 ± 64.9	690.7 ± 55.9	537.5 ± 27.0*	445.8 ± 13.5**
Sperm (10^6/cauda epididymis)	24.45 ± 0.95	18.40 ± 0.41**	16.48 ± 0.72**	14.64 ± 0.25**

* Significantly different (P≤0.05) from the chamber control group by Dunnett's test (left testis weights), Dunn's test (spermatid heads/mg testis measurements), or Shirley's test (sperm/mg cauda epididymis measurements).
** Significantly different (P≤0.01) from the chamber control group by Dunnett's test (left cauda epididymis weights) or Shirley's test (sperm/mg cauda epididymis and sperm/cauda epididymis measurements).
[a] Data are presented as mean ± standard error.
[b] n=9

TABLE E5
Estrous Cycle Characterization for Female Mice in the 3-Month Inhalation Study of α-Pinene[a]

	Chamber Control	100 ppm	200 ppm	400 ppm
Number weighed at necropsy	10	10	10	10
Necropsy body wt (g)	31.5 ± 0.6	31.5 ± 1.1	30.7 ± 0.6	30.6 ± 0.5
Proportion of regular cycling females[b]	9/10	8/9	9/10	8/9
Estrous cycle length (days)	3.91 ± 0.05	4.04 ± 0.15[c]	3.96 ± 0.07	3.82 ± 0.11[d]
Estrous stages[e] (% of cycle)				
Diestrus	25.0	32.5	26.7	27.8
Proestrus	0.0	0.0	0.0	0.0
Estrus	50.8	45.8	50.0	50.0
Metestrus	24.2	21.7	23.3	22.2

[a] Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[b] Number of females with a regular cycle/number of females cycling
[c] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.
[d] n=9
[e] By multivariate analysis of variance, exposed females do not differ significantly from the chamber control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the chamber control group and each exposed group indicated exposed females did not spend significantly more time in the estrous stages than did the chamber control females.

Concentration (ppm)																							
0								D	E	E	E	M	D	E	E	M	D	E	E				
0											E	M	D	E	E	M	D	E	E	M	D	E	
0											E	M	D	E	E	M	D	E	E	M	D	E	
0									D	E	E	M	D	E	E	M	D	E	E	M			
0										E	E	M	D	E	E	M	D	E	E	M	D		
0										E	E	M	D	E	E	M	D	E	E	M	D		
0										E	E	M	D	E	E	M	D	E	E	M	D		
0										E	E	M	D	E	E	M	D	E	E	M	D		
0										E	E	M	D	E	E	M	D	E	E	M	D		
0											E	M	D	E	E	M	D	E	E	M	D	E	
100									D	E	E	M	D	E	E	M	D	E	E	M			
100											E	M	D	E	E	M	D	E	E	M	D	E	
100										E	E	M	D	E	E	M	D	E	E	M	D		
100										E	E	M	D	E	E	M	D	E	E	M	D		
100									E	E	M	D	D	E	E	E	M	D	E	E			
100										E	M	D	D	E	E	M	D	E	E	M	D		
100								M	D	E	E	M	D	E	E	M	D	E	E				
100		M	D	D	D	D	D	D	D	E	E	D	D										
100							D	D	D	E	E	M	D	E	E	M	D	E					
100											E	M	D	E	E	M	D	E	E	M	D	E	
200									D	E	E	M	D	E	E	M	D	E	E	M			
200										E	E	M	D	E	E	M	D	E	E	M	D		
200									D	E	E	M	D	E	E	E	M	D	E	E			
200											E	M	D	E	E	M	D	E	E	M	D	D	
200									D	E	E	M	D	E	E	M	D	E	E	M			
200											E	M	D	E	E	M	D	E	E	M	D	E	
200											E	M	D	E	E	M	D	E	E	M	D	E	
200									D	E	E	D	D	E	E	M	D	E	E	M			
200											E	M	D	E	E	M	D	E	E	M	D	E	
200								M	D	E	E	M	D	E	E	M	D	E	E				
400									D	E	E	M	D	E	E	M	D	E	E	M			
400								M	D	E	E	M	D	E	E	D	D	E	E				
400										E	E	M	D	E	E	M	D	E	E	M	D		
400										E	E	M	D	E	E	M	D	E	E	M	D		
400											E	M	D	E	E	M	D	E	E	M	D	E	
400											E	M	D	E	E	M	D	E	E	M	D	E	
400			D	D	D	D	E	E	E	E	E	M	D	E									
400								M	D	E	E	M	D	E	E	M	D	E	E				
400										E	E	M	D	E	E	M	D	E	E	M	D		

FIGURE E2
Individual Vaginal Cytology Plots for Female Mice in the 3-Month Inhalation Study of α-Pinene
D = diestrus, E = estrus, M = metestrus. Cytology is aligned based on the second estrus observation.

TABLE E6
Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Mice Administered α-Pinene by Inhalation for 3 Months

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	0.12	
Overall Tests	100 ppm vs. Chamber Controls	0.033	
Overall Tests	200 ppm vs. Chamber Controls	1	
Overall Tests	400 ppm vs. Chamber Controls	0.191	
Extended Estrus	Overall	0.969	
Extended Estrus	100 ppm vs. Chamber Controls	1	
Extended Estrus	200 ppm vs. Chamber Controls	1	
Extended Estrus	400 ppm vs. Chamber Controls	0.509	
Extended Diestrus	Overall	0.994	
Extended Diestrus	100 ppm vs. Chamber Controls	0.703	
Extended Diestrus	200 ppm vs. Chamber Controls	1	
Extended Diestrus	400 ppm vs. Chamber Controls	0.995	
Extended Metestrus	Overall	1	
Extended Metestrus	100 ppm vs. Chamber Controls	1	
Extended Metestrus	200 ppm vs. Chamber Controls	1	
Extended Metestrus	400 ppm vs. Chamber Controls	1	
Extended Proestrus	Overall	1	
Extended Proestrus	100 ppm vs. Chamber Controls	1	
Extended Proestrus	200 ppm vs. Chamber Controls	1	
Extended Proestrus	400 ppm vs. Chamber Controls	1	
Skipped Estrus	Overall	1	
Skipped Estrus	100 ppm vs. Chamber Controls	1	
Skipped Estrus	200 ppm vs. Chamber Controls	1	
Skipped Estrus	400 ppm vs. Chamber Controls	1	
Skipped Diestrus	Overall	1	
Skipped Diestrus	100 ppm vs. Chamber Controls	1	
Skipped Diestrus	200 ppm vs. Chamber Controls	1	
Skipped Diestrus	400 ppm vs. Chamber Controls	1	
Summary of Significant Groups			
Overall Tests	100 ppm vs. Chamber Controls	0.033	

[a] N means that the treated group had a lower probability of transitioning to the relevant abnormal state (extended estrus, extended diestrus, extended metestrus, extended proestrus, skipped estrus, or skipped diestrus) than did the chamber control group.

APPENDIX F
CHEMICAL CHARACTERIZATION AND GENERATION OF CHAMBER CONCENTRATIONS

CHEMICAL CHARACTERIZATION AND GENERATION OF CHAMBER CONCENTRATIONS

PROCUREMENT AND CHARACTERIZATION OF α-PINENE

α-Pinene was obtained from Millennium Specialty Chemicals (Jacksonville, FL) in one lot (4KB705) that was used in the 2-week and 3-month studies. Identity and purity analyses were conducted by the study laboratory at Battelle Toxicology Northwest (Richland, WA), Chemir Analytical Services (Maryland Heights, MO), Galbraith Laboratories, Inc. (Knoxville, TN), and Huffman Laboratories, Inc. (Golden, CO). Reports on analyses performed in support of the α-pinene studies are on file at the National Institute of Environmental Health Sciences.

Lot 4KB705 of the chemical, a colorless oily liquid with a strong piney odor, was identified as α-pinene by Chemir Analytical Services using infrared (IR) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. All spectra were consistent with the literature reference spectra (*Aldrich*, 1993, 1997) of α-pinene. Representative IR and ^{1}H-NMR spectra are presented in Figures F1 and F2, respectively.

Chemir Analytical Services determined the moisture content of lot 4KB705 using Karl Fischer titration and Galbraith Laboratories, Inc., and Huffman Laboratories, Inc., performed elemental analyses. The purity of lot 4KB705 was determined by the study laboratory using gas chromatography (GC) by systems A through D with flame ionization detection (FID) or mass spectrometry (MS) detection (Table F1).

For lot 4KB705, Karl Fischer titration indicated a water content of 27 ppm. Elemental analyses for carbon, hydrogen, and nitrogen were in agreement with the theoretical values for α-pinene. GC/FID by system A indicated one major peak accounting for approximately 96% of the total integrated peak area and three impurity peaks with areas exceeding 0.1% of the total peak area; two of these peaks matched the retention times for prepared standards of camphene (1.77%) and β-pinene (1.73%). GC/MS by system B identified the third impurity as tricyclene (0.51%). Enantiomeric composition analysis using GC/FID system D with a chiral separation column indicated that the lot was 69% (+)-α-pinene and 31% (–)-α-pinene. The overall purity of lot 4KB705 was determined to be approximately 96%. Analysis using GC/MS by system C indicated that approximately 15 to 16 ppm butylated hydroxy toluene (BHT), a free radical scavenger, was present in the lot to prevent oxidation of α-pinene.

To ensure stability, the bulk chemical was stored at 17° C in the original shipping containers (55-gallon metal drums). Periodic reanalyses of the bulk chemical were performed during the 2-week and 3-month studies by the study laboratory using GC/MS by system B, and no degradation of the bulk chemical was detected.

VAPOR GENERATION AND EXPOSURE SYSTEM

A diagram of the α-pinene vapor generation and delivery system used in the studies is shown in Figure F3. The design of the system was influenced by the relatively high boiling point for α-pinene (approximately 156° C) and the need to reach relatively high concentrations. Therefore, the vapor transport lines and all dilution air were heated. α-Pinene was held in an 8-gallon stainless-steel chemical reservoir. α-Pinene was pumped through a preheater (for the 2-week studies) and into a heated glass column filled with glass beads that increased the surface area for vaporization. Heated nitrogen entered the column from below and assisted in vaporizing the chemical while conveying it into a short distribution manifold. Concentration in the manifold was determined by the chemical pump rate, nitrogen flow rate, and dilution air flow rate. The pressure in the distribution manifold was kept fixed to ensure constant flow through the manifold and into all chambers as the flow of vapor to each chamber was adjusted.

Metering valves at the manifold controlled flow to each chamber through individual Teflon® delivery lines that carried the vapor from the manifold to three-way exposure valves at the chamber inlets. The exposure valves diverted vapor delivery to exposure chamber exhaust until the generation system was stable and exposures were ready to proceed. To initiate exposure, the chamber exposure valves were rotated to allow the α-pinene vapor to flow to each exposure chamber inlet duct where it was further diluted with filtered, conditioned air to achieve the desired exposure concentration.

The study laboratory designed the inhalation exposure chamber (Lab Products, Inc., Seaford, DE) so that uniform vapor concentrations could be maintained throughout the chamber with the catch pans in place. The total active mixing volume of each chamber was 1.7 m^3. A condensation particle detector (Model 3022A, TSI, Inc., St. Paul, MN) was used with and without animals in the exposure chambers. No particle counts above the minimum resolvable level (approximately 200 particles/cm^3) were detected.

VAPOR CONCENTRATION MONITORING

Summaries of the chamber vapor concentrations are given in Tables F2 and F3. Chamber and room concentrations of α-pinene were monitored by an on-line gas chromatograph (system E, Table F1). Samples were drawn from each exposure chamber approximately every 20 minutes during each 6-hour exposure period. A 16-port stream select valve (VALCO Instruments Company, Houston, TX) directed a continuous stream of sampled atmosphere to a six-port sampling valve (VALCO Instruments Company) with a 1.0 mL sample loop, housed in a dedicated valve oven at 175° C. A vacuum regulator maintained a constant vacuum in the sample loop to compensate for variations in sample line pressure. An in-line flow meter between the vacuum regulator and the gas chromatograph allowed digital measurement of sample flow.

The on-line gas chromatograph was checked throughout the day for instrument drift against an on-line standard vapor of α-pinene in nitrogen supplied by a standard generator (Kin-Tek; Precision Calibration Systems, La Marque, TX). The on-line gas chromatograph was recalibrated as required to meet acceptance criteria. Calibration was performed by a comparison of chamber concentration data to data from grab samples that were collected with activated coconut charcoal gas sampling tubes (ORBO™-32; Supelco, Inc., Bellefonte, PA), extracted with toluene containing butylbenzene as an internal standard, and analyzed using an off-line gas chromatograph (system F). Known volumes of chamber atmosphere were sampled at a constant flow rate ensured by a calibrated critical orifice. The off-line gas chromatograph was calibrated with gravimetrically prepared standards of α-pinene containing butylbenzene as an internal standard in toluene.

CHAMBER ATMOSPHERE CHARACTERIZATION

Buildup and decay rates for chamber vapor concentrations were determined with and without animals present in the chambers. At a chamber airflow rate of 15 air changes per hour, the theoretical value for the time to achieve 90% of the target concentration after the beginning of vapor generation (T_{90}) and the time for the chamber concentration to decay to 10% of the target concentration after vapor generation was terminated (T_{10}) was approximately 9.4 minutes. For rats and mice in the 2-week studies, T_{90} values ranged from 8 to 9 minutes with animals present and T_{10} values ranged from 9 to 10 minutes with animals present. For rats and mice in the 3-month studies, T_{90} values ranged from 8 to 9 minutes without animals present and from 9 to 12 minutes with animals; T_{10} values ranged from 8 to 10 minutes without animals present and from 9 to 10 minutes with animals. A T_{90} value of 12 minutes was selected for the 2-week studies and a T_{90} value of 10 minutes was selected for the 3-month studies.

The uniformity of vapor concentration in the inhalation exposure chambers without animals present was evaluated before the 3-month studies began; in addition, concentration uniformity with animals present in the chambers was measured once during the 2-week and 3-month studies. The vapor concentration was measured using the on-line gas chromatograph (system E, Table F1) with the stream-selection valve fixed in one position to allow continuous monitoring from a single input line. During the studies, concentrations were measured at 12 chamber positions, one in front and one in back for each of the six possible animal cage unit positions per chamber. Chamber concentration uniformity was maintained throughout the studies.

The persistence of α-pinene in the chambers after vapor delivery ended was determined by monitoring the vapor concentration overnight in the 1,600 ppm chamber in the 2-week studies and the 400 ppm chamber in the 3-month studies. In the 2-week studies, the concentration decreased to 1% of the target concentration within 22 minutes with animals present. In the 3-month studies, the concentration decreased to 1% of the target concentration within 21 minutes with animals present and within 22 minutes without animals present.

Samples of the test atmosphere from the distribution lines and low and high exposure concentration chambers were collected at the beginning and end of one generation day during the 2-week studies and at the beginning and end of

one generation day prior to the 3-month studies and one generation day during the 3-month studies. Atmosphere samples were collected with adsorbent gas sampling tubes containing activated coconut charcoal (ORBO™-32; Supelco, Inc.), followed by a tube containing silica gel (ORBO™-52; Supelco, Inc.) and extracted with methylene chloride. Additional samples were collected from the generator reservoir, and all of the samples were analyzed using GC/FID by system A (with a final temperature of 260° C for the 3-month studies) to measure the stability and purity of α-pinene in the generation and delivery system. To assess whether impurities or degradation products coeluted with α-pinene or the solvent, a second GC/FID analysis of the samples was performed using a polar column capable of resolving compounds with similar boiling points and polarities (system G; final temperature was 260° C for the 3-month studies). Analyses of BHT content and enantiomeric ratio in the generation and delivery system samples were conducted using GC/MS by system C and GC/FID by system D, respectively.

No evidence of degradation of α-pinene was noted in any part of the exposure system. Three impurity peaks with areas greater than 0.1% of the total peak area were consistently detected in atmosphere and generator reservoir samples collected during the 2-week and 3-month studies. These peaks matched the retention times for prepared standards of tricyclene, camphene, and β-pinene and had area percent values similar to those measured during the initial bulk purity analyses of the test chemical. Using the polar column, no additional impurities were detected in any of the atmosphere or generator reservoir samples collected during the studies. Values for BHT content and enantiomeric ratio in the samples were similar to those determined in the initial purity assessments of lot 4KB705.

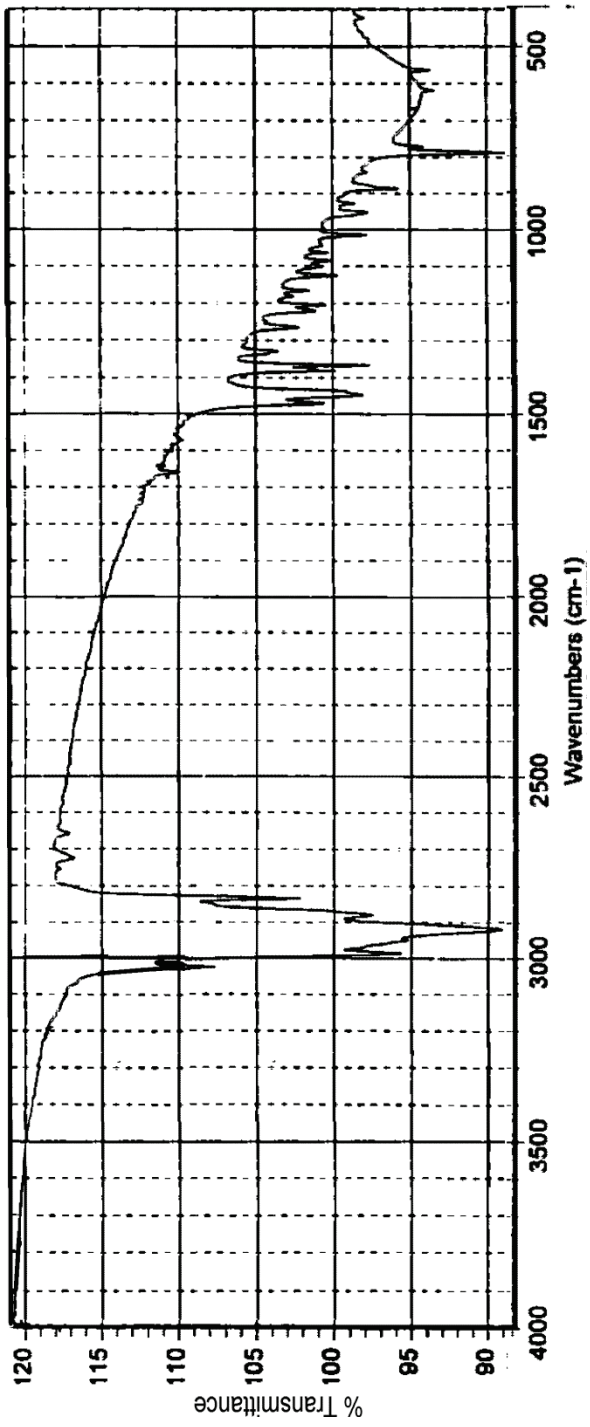

FIGURE F1
Infrared Absorption Spectrum of α-Pinene

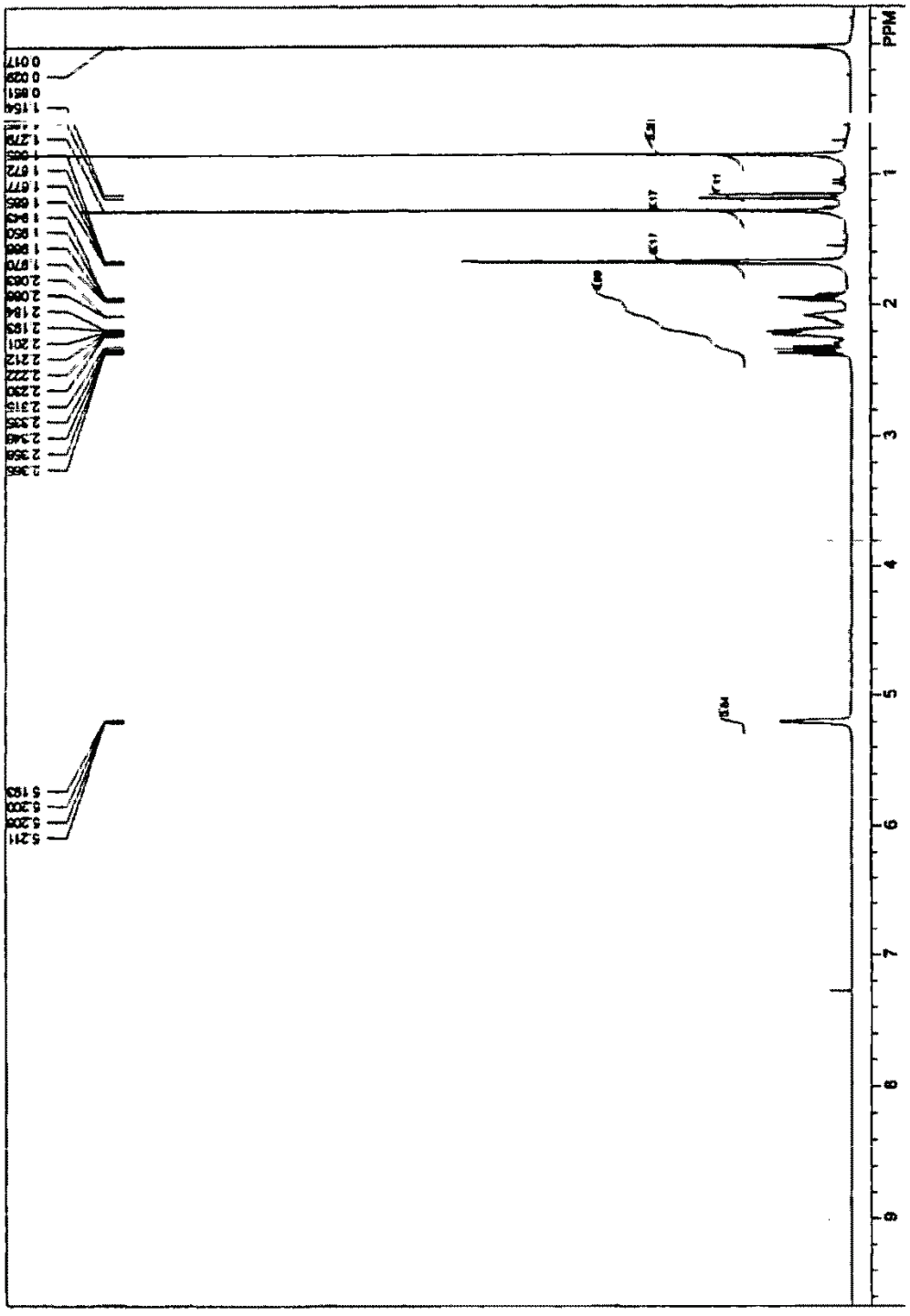

FIGURE F2
^{1}H-Nuclear Magnetic Resonance Spectrum of α-Pinene

TABLE F1
Gas Chromatography Systems Used in the Inhalation Studies of α-Pinene[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A			
Flame ionization	DB-5 (J&W Scientific, Folsom, CA) or Rtx-5 (Restek, Bellefonte, PA), 30 m × 0.53 mm, 1.5-µm film thickness	Helium at 6 psi head pressure	40° C for 3 minutes, then 4° C/minute to 300° C
System B			
Mass spectrometry	DB-5, 30 m × 0.25 mm, 1.0-µm film thickness (J&W Scientific)	Helium at 6 psi head pressure	40° C for 3 minutes, then 4° C/minute to 260° C
System C			
Mass spectrometry	DB-5, 30 m × 0.25 mm, 1.0-µm film thickness (J&W Scientific)	Helium at 6 psi head pressure	90° C for 3 minutes, then 8° C/minute to 240° C
System D			
Flame ionization	CycloSil-B, 30 m × 0.25 mm, 0.25-µm film thickness (J&W Scientific)	Helium at 16 psi head pressure	60° C for 0.5 minutes, then 5° C/minute to 105° C, then 20° C/minute to 160° C
System E			
Flame ionization	DB-5, 15 m × 0.53 mm, 1.5-µm film thickness (J&W Scientific)	Nitrogen at 25 mL/minute	Isothermal at 85° C
System F			
Flame ionization	DB-5, 30 m × 0.53 mm, 1.5-µm film thickness (J&W Scientific)	Helium at 6 psi head pressure	60° C for 1 minute, then 8° C/minute to 110° C, then 15° C/minute to 200° C
System G			
Flame ionization	DB-WAX, 30 m × 0.53 mm, 1.0-µm film thickness (J&W Scientific)	Helium at 6 psi head pressure	40° C for 3 minutes, then 4° C/minute to 300° C

[a] The gas chromatographs were manufactured by Hewlett-Packard (Palo Alto, CA).

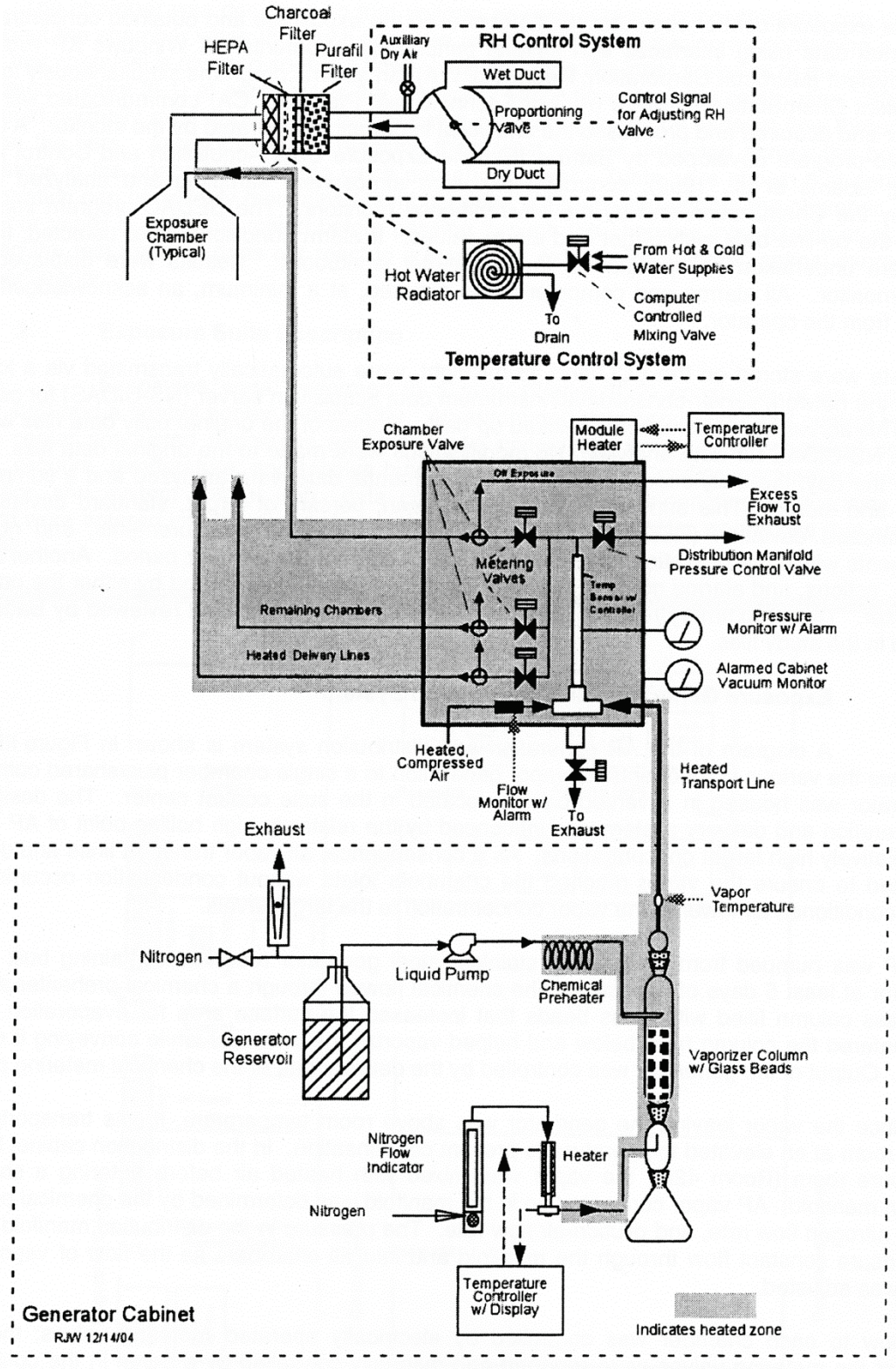

FIGURE F3
Schematic of the Vapor Generation and Delivery System in the Inhalation Studies of α-Pinene

TABLE F2
Summary of Chamber Concentrations in the 2-Week Inhalation Studies of α-Pinene

	Target Concentration (ppm)	Total Number of Readings	Average Concentration[a] (ppm)
Rat Chambers			
	100	205	99.6 ± 1.4
	200	207	200 ± 1
	400	206	404 ± 4
	800	190	794 ± 37
	1,600	16	1,540 ± 130
Mouse Chambers			
	100	223	99.8 ± 1.6
	200	225	200 ± 1
	400	224	404 ± 4
	800	190	794 ± 37
	1,600	16	1,540 ± 129

[a] Mean ± standard deviation

TABLE F3
Summary of Chamber Concentrations in the 3-Month Inhalation Studies of α-Pinene

	Target Concentration (ppm)	Total Number of Readings	Average Concentration[a] (ppm)
Rat Chambers			
	25	1,225	24.9 ± 1.1
	50	1,212	49.8 ± 0.8
	100	1,227	99.6 ± 1.4
	200	1,256	200 ± 5
	400	1,264	401 ± 6
Mouse Chambers			
	25	1,265	24.9 ± 1.1
	50	1,250	49.8 ± 0.8
	100	1,265	99.6 ± 1.4
	200	1,296	200 ± 4
	400	1,304	401 ± 7

[a] Mean ± standard deviation

APPENDIX G
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NTP-2000 RAT AND MOUSE RATION

TABLE G1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix[a]	0.5
Mineral premix[b]	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

[a] Wheat middlings as carrier
[b] Calcium carbonate as carrier

TABLE G2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a] Per kg of finished product

TABLE G3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	14.4 ± 0.62	13.7 – 15.3	5
Crude fat (% by weight)	8.0 ± 0.21	7.8 – 8.3	5
Crude fiber (% by weight)	9.3 ± 0.34	8.9 – 9.8	5
Ash (% by weight)	4.7 ± 0.18	4.6 – 5.0	5
Amino Acids (% of total diet)			
Arginine	0.783 ± 0.070	0.670 – 0.970	22
Cystine	0.220 ± 0.024	0.150 – 0.250	22
Glycine	0.701 ± 0.041	0.620 – 0.800	22
Histidine	0.352 ± 0.077	0.270 – 0.680	22
Isoleucine	0.546 ± 0.044	0.430 – 0.660	22
Leucine	1.095 ± 0.067	0.960 – 1.240	22
Lysine	0.711 ± 0.114	0.310 – 0.860	22
Methionine	0.409 ± 0.046	0.260 – 0.490	22
Phenylalanine	0.628 ± 0.040	0.540 – 0.720	22
Threonine	0.505 ± 0.043	0.430 – 0.610	22
Tryptophan	0.150 ± 0.028	0.110 – 0.200	22
Tyrosine	0.401 ± 0.061	0.280 – 0.540	22
Valine	0.665 ± 0.043	0.550 – 0.730	22
Essential Fatty Acids (% of total diet)			
Linoleic	3.95 ± 0.259	3.49 – 4.55	22
Linolenic	0.30 ± 0.032	0.21 – 0.35	22
Vitamins			
Vitamin A (IU/kg)	4,276 ± 67	3,230 – 5,080	5
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	80.6 ± 22.03	27.0 – 124.0	22
Thiamine (ppm)[b]	7.5 ± 0.73	6.6 – 8.6	5
Riboflavin (ppm)	7.6 ± 2.89	4.20 – 17.50	22
Niacin (ppm)	78.9 ± 9.08	66.4 – 98.2	22
Pantothenic acid (ppm)	26.9 ± 12.63	17.4 – 81.0	22
Pyridoxine (ppm)[b]	9.54 ± 1.99	6.44 – 13.7	22
Folic acid (ppm)	1.62 ± 0.48	1.15 – 3.27	22
Biotin (ppm)	0.32 ± 0.10	0.2 – 0.704	22
Vitamin B_{12} (ppb)	53.6 ± 39.6	18.3 – 174.0	22
Choline (ppm)[b]	2,846 ± 485	1,820 – 3,790	22
Minerals			
Calcium (%)	0.961 ± 0.045	0.924 – 1.030	5
Phosphorus (%)	0.558 ± 0.018	0.535 – 0.576	5
Potassium (%)	0.666 ± 0.030	0.626 – 0.733	22
Chloride (%)	0.386 ± 0.039	0.300 – 0.474	22
Sodium (%)	0.189 ± 0.016	0.160 – 0.222	22
Magnesium (%)	0.216 ± 0.062	0.185 – 0.490	22
Sulfur (%)	0.170 ± 0.029	0.116 – 0.209	14
Iron (ppm)	186 ± 39.2	135 – 311	22
Manganese (ppm)	51.4 ± 10.28	21.0 – 73.1	22
Zinc (ppm)	53.4 ± 8.46	43.3 – 78.5	22
Copper (ppm)	7.01 ± 2.562	3.21 – 16.3	22
Iodine (ppm)	0.503 ± 0.206	0.158 – 0.972	22
Chromium (ppm)	0.694 ± 0.276	0.330 – 1.380	22
Cobalt (ppm)	0.256 ± 0.164	0.098 – 0.864	20

[a] From formulation
[b] As hydrochloride (thiamine and pyridoxine) or chloride (choline)

TABLE G4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.22 ± 0.036	0.19 – 0.28	5
Cadmium (ppm)	0.05 ± 0.004	0.04 – 0.05	5
Lead (ppm)	0.10 ± 0.020	0.08 – 0.12	5
Mercury (ppm)	<0.02		5
Selenium (ppm)	0.24 ± 0.034	0.19 – 0.26	5
Aflatoxins (ppb)	<5.00		5
Nitrate nitrogen (ppm)[c]	8.60 ± 0.631	7.89 – 9.40	5
Nitrite nitrogen (ppm)[c]	3.78 ± 2.200	0.30 – 5.81	5
BHA (ppm)[d]	<1.0		5
BHT (ppm)[d]	<1.0		5
Aerobic plate count (CFU/g)	10 ± 0	10	5
Coliform (MPN/g)	3.0 ± 0	3.0	5
Escherichia coli (MPN/g)	<10		5
Salmonella (MPN/g)	Negative		5
Total nitrosamines (ppb)[e]	4.8 ± 1.41	3.2 – 6.1	5
N-Nitrosodimethylamine (ppb)[e]	2.9 ± 1.20	3.9 – 1.4	5
N-Nitrosopyrrolidine (ppb)[e]	1.9 ± 0.26	1.5 – 2.2	5
Pesticides (ppm)			
α-BHC	<0.01		5
β-BHC	<0.02		5
γ-BHC	<0.01		5
δ-BHC	<0.01		5
Heptachlor	<0.01		5
Aldrin	<0.01		5
Heptachlor epoxide	<0.01		5
DDE	<0.01		5
DDD	<0.01		5
DDT	<0.01		5
HCB	<0.01		5
Mirex	<0.01		5
Methoxychlor	<0.05		5
Dieldrin	<0.01		5
Endrin	<0.01		5
Telodrin	<0.01		5
Chlordane	<0.05		5
Toxaphene	<0.10		5
Estimated PCBs	<0.20		5
Ronnel	<0.01		5
Ethion	<0.02		5
Trithion	<0.05		5
Diazinon	<0.10		5
Methyl chlorpyrifos	0.176 ± 0.156	0.025 – 0.356	5
Methyl parathion	<0.02		5
Ethyl parathion	<0.02		5
Malathion	0.334 ± 0.094	0.233 – 0.461	5
Endosulfan I	<0.01		5
Endosulfan II	<0.01		5
Endosulfan sulfate	<0.03		5

[a] Samples was irradiated. CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride
[b] For values less than the limit of detection, the detection limit is given.
[c] Sources of contamination: alfalfa, grains, and fish meal
[d] Sources of contamination: soy oil and fish meal
[e] All values were corrected for percent recovery

APPENDIX H
SENTINEL ANIMAL PROGRAM

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or exposed animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of compounds.

Blood samples were collected from each animal and allowed to clot, and the serum was separated. All samples were processed appropriately and tested in-house or sent to BioReliance Corporation (Rockville, MD) (3-month study termination samples) for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five animals per sex per time point.

	Method /Test	Time of Collection
RATS		
2-Week Study	**In-House Antibody Testing**	
	Mycoplasma pulmonis	Study termination
	PVM (Pneumonia virus of mice)	Study termination
	RCV/SDA (Rat coronavirus/sialodacryoadenitis virus)	Study termination
	RPV (Rat parvovirus)	Study termination
	Sendai	Study termination
13-Week Study	**In-House Antibody Testing**	
	M. pulmonis	1 week
	PVM	1 week
	RCV/SDA	1 week
	RPV	1 week
	Sendai	1 week
	ELISA	
	PVM	Study termination
	RCV/SDA	Study termination
	Sendai	Study termination
	Immunofluorescence Assay	
	Parvovirus	Study termination

MICE

2-Week Study	**In-House Antibody Testing**	
	GDVII (Theiler's murine encephalomyelitis virus)	Study termination
	MHV (Mouse hepatitis virus)	Study termination
	MPV (Mouse parvovirus)	Study termination
	M. pulmonis	Study termination
	PVM	Study termination
	Sendai	Study termination
13-Week Study	**In-House Antibody Testing**	
	GDVII	1 week
	MHV	1 week
	MPV	1 week
	M. pulmonis	1 week
	PVM	1 week
	Sendai	1 week
	ELISA	
	Ectromelia virus	Study termination
	EDIM (epizootic diarrhea of infant mice)	Study termination
	GDVII	Study termination
	LCM (lymphocytic choriomeningitis virus)	Study termination
	MAd-FL (Mouse adenovirus)	Study termination
	MHV	Study termination
	MMV VP2 (Mouse minute virus viral protein 2)	Study termination
	MPV VP2 (Mouse parvovirus viral protein 2)	Study termination
	PVM	Study termination
	Reovirus	Study termination
	Sendai	Study termination

RESULTS

All test results were negative.